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Transcriptional Control and Population Dynamics of Antiviral CD8+ T Cell Responses

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Transcriptional Control and Population Dynamics of Antiviral CD8⁺ T Cell Responses

Abstract

Cytotoxic lymphocytes are central components of cellular immune responses to intracellular pathogens and malignancy. The transcriptional programs that support proper population dynamics for lifelong immunity are incompletely understood. Two T-box transcription factors, T-bet and Eomesodermin (Eomes), have critical roles in the development of natural killer cells and the differentiation of CD8⁺ T cells in response to acutely resolved infections. In both cases, these two factors support distinct but complementary cellular populations. In this thesis, we first used a recently generated reagent to examine and separate cellular populations with differential Eomes expression. We found that, while Eomes expression does not identify effector CD8⁺ T cells with enhanced cytotoxic potential, early Eomes expression does correlate with more efficient formation of long-lived, self-renewing central memory CD8⁺ T cells. This validated tool was then employed in our investigation into the population dynamics of CD8⁺ T cell responses during a chronic viral infection. While memory lymphocytes maintain lifelong immunity by slow self-renewal, chronic infections strain the regenerative capacity of antiviral T lymphocyte populations, leading to failure in long-term immunity. The cellular and molecular events controlling the regenerative capacity during chronic infection, however, are unknown. We demonstrate that two distinct states of virus-specific CD8⁺ T cells exist in chronically infected mice and humans. The opposing properties of renewal and differentiation of these CD8⁺ T cell populations are supported by the differential expression of T-box transcription factors, which cooperatively maintain the pool of antiviral CD8⁺ T cells during chronic viral infection. T-bet^{hi} cells have low intrinsic turnover but proliferate in response to persisting antigen, giving rise to Eomes^{hi} terminal progeny. Genetic elimination of either subset results in failure to control chronic infection, suggesting that imbalance in differentiation and renewal could underlie the collapse of immunity in humans with chronic infections. Furthermore, this work demonstrates new roles for T-bet and Eomes in CD8⁺ T cells regarding self-renewal and terminal differentiation, highlighting how T-box transcription factors can operate in exquisitely context-dependent manners.

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OF ANTIVIRAL CD8⁺ T CELL RESPONSES

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Michael Alexander Paley

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ABSTRACT

TRANSCRIPTIONAL CONTROL AND POPULATION DYNAMICS OF ANTIVIRAL CD8⁺ T CELL RESPONSES

Michael A. Paley

E. John Wherry, Ph.D.

Cytotoxic lymphocytes are central components of cellular immune responses to intracellular pathogens and malignancy. The transcriptional programs that support proper population dynamics for lifelong immunity are incompletely understood. Two T-box transcription factors, T-bet and Eomesodermin (Eomes), have critical roles in the development of natural killer cells and the differentiation of CD8⁺ T cells in response to acutely resolved infections. In both cases, these two factors support distinct but complementary cellular populations. In this thesis, we first used a recently generated reagent to examine and separate cellular populations with differential Eomes expression. We found that, while Eomes expression does not identify effector CD8⁺ T cells with enhanced cytotoxic potential, early Eomes expression does correlate with more efficient formation of long-lived, self-renewing central memory CD8⁺ T cells. This validated tool was then employed in our investigation into the population dynamics of CD8⁺ T cell responses during a chronic viral infection. While memory lymphocytes maintain lifelong immunity by slow self-renewal, chronic infections strain the regenerative capacity of antiviral T lymphocyte populations, leading to failure in long-term immunity. The cellular and molecular events controlling the regenerative capacity during chronic infection,

however, are unknown. We demonstrate that two distinct states of virus-specific CD8⁺ T cells exist in chronically infected mice and humans. The opposing properties of renewal and differentiation of these CD8⁺ T cell populations are supported by the differential expression of T-box transcription factors, which cooperatively maintain the pool of antiviral CD8⁺ T cells during chronic viral infection. T-bet^{hi} cells have low intrinsic turnover but proliferate in response to persisting antigen, giving rise to Eomes^{hi} terminal progeny. Genetic elimination of either subset results in failure to control chronic infection, suggesting that imbalance in differentiation and renewal could underlie the collapse of immunity in humans with chronic infections. Furthermore, this work demonstrates new roles for T-bet and Eomes in CD8⁺ T cells regarding self-renewal and terminal differentiation, highlighting how T-box transcription factors can operate in exquisitely context-dependent manners.

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CHAPTER 1: Introduction

1.1 Viral challenges to human health

Viral infections are a common challenge to the mammalian immune system. While many viral infections induce minimal disease in immunocompetent individuals, some viruses pose significant threats to worldwide health. For example in the United States, seasonal influenza averages 20,000 deaths every year as a result of acute infection (CDC, 2010). In contrast, other viruses, such as human immunodeficiency virus (HIV) or hepatitis C virus (HCV), establish a chronic infection and elicit disease over the ensuing years, each averaging 15,000 annual deaths (CDC, 2011; Ly et al., 2012). Therapies against HCV, while potentially curative, display low efficacy (Fried et al., 2002; Manns et al., 2001; McHutchison et al., 1998; McHutchison et al., 2009; Welsch et al., 2012). In contrast, current medical interventions for HIV infection are not geared towards viral elimination, but rather are limited to reducing viral replication (Piacenti, 2006). Consequently, investigators continue to pursue other approaches that have the potential to synergize with or potentially replace current medical therapies. In particular, prophylactic or therapeutic interventions based on antiviral immune responses have demonstrated potential in this regard (Barber et al., 2006; Buchbinder et al., 2008; Diskin et al., 2011; Hansen et al., 2011; Rerks-Ngarm et al., 2009; Scheid et al., 2009; Scheid et al., 2011; Velu et al., 2009; Wei et al., 2010; Zhou et al., 2010). Nevertheless, it remains to be defined which components of the adaptive immune system are required to generate effective and broad immunity to each of these individual pathogens.

1.2 Adaptive immune responses

While humoral immunity can provide an effective obstacle to establishment of a viral infection through antibody-mediated neutralization, clearance of an invading microbe depends on an effective response by the cellular arm of the adaptive immune system. In particular, CD8⁺ T cells fulfill a critical role due to their ability to recognize virally infected cells. This recognition is mediated by interactions between the T cell receptor (TCR) and components of degraded foreign viral proteins (8-10 amino acids in length) presented by the major histocompatibility complex (MHC) (Rock and Goldberg, 1999). In this way, CD8⁺ T cells can recognize virally infected cells that may not display outward signs of infection.

The importance of CD8⁺ T cell responses in controlling viral replication and subsequent disease has been implicated through examination of immunodeficient patients with susceptibility to viral infection and through genome-wide association studies after viral infection. For example, genetic mutations in the effector molecule perforin (discussed below) can impair both CD8⁺ T cell function and immune clearance of viral infection, leading to the inflammatory disorder termed hemophagocytic lymphohistiocytosis (HLH) (Jordan et al., 2004; Kogawa et al., 2002; Pachlopnik Schmid et al., 2009; Stepp et al., 1999). In addition, mutations in the receptor for IFN-gamma (discussed below), a major effector cytokine of CD8⁺ T cells, have been associated with severe viral infections, including herpes, parainfluenza, and respiratory syncytial viruses (Dorman et al., 1999). Similar to inherited or spontaneous mutations that lead to immunodeficiencies early in life, acquired immunosuppression also illustrates the importance of CD8⁺ T cells in response to viral infections. For example, immunocompromised individuals due to HIV infection are at risk for clinical reactivation

of JC polyomavirus and subsequent progressive multifocal leukoencephalopathy (PML). In this setting, disease resolution was strongly associated with detectable antiviral CD8⁺ T cell responses (Du Pasquier et al., 2004). Thus, genetically impaired or immunologically poor CD8⁺ T cell responses have been suggested to lead to a variety of human disease states after viral infection.

In parallel to these “experiments of nature,” genomic sequencing of polymorphisms has provided insight into individual genes that modulate control of or susceptibility to viral infection. For example during HIV infection, while several genes associated with co-receptor binding and viral entry, such as CCR5 and beta chemokines (CCL3-CCL5), have been linked to superior clinical outcomes, a number of MHC alleles have been associated with improved control of HIV replication, consistent with the hypothesis that CD8⁺ T cell responses are an essential component of the antiviral response (Chapman and Hill, 2012). Similarly, MHC alleles have also been associated with clearance or susceptibility to hepatitis B virus (HBV) (He et al., 2006) or enterovirus infection (Chang et al., 2008). Thus, the ability of CD8⁺ T cells to recognize virally infected target cells is associated with clinical protection against viral infection.

1.3 CD8⁺ T cell responses

Maintenance of virus-specific CD8⁺ T cell responses poses a unique challenge. Unlike other tissues with high rates of cellular replacement from long-lived progenitors, the CD8⁺ T cells that comprise antiviral responses may be genetically unique due to rearrangement of the TCR locus during thymic development (Koch and Radtke, 2011). In addition, these cells may have a low probability of replacement after age-associated thymic involution (Lynch et al., 2009). As a consequence, antiviral CD8⁺ T cells illustrate

a heightened tension between renewal (to preserve the unique TCR rearrangement) and terminal differentiation (to effectively mount an antiviral response).

1.3.1 Initiation of antiviral CD8⁺ T cell responses

After exposure to antigen, a naive CD8⁺ T cell initiates a phase of proliferation and differentiation to generate a large population of antiviral effector cells (Butz and Bevan, 1998; Murali-Krishna et al., 1998). CD8⁺ T cells mediate viral clearance through a variety of mechanisms. The most well-known effector function of “killer” T cells is the destruction of virally infected target cells. This cytotoxicity can be mediated by at least two separate mechanisms. The first is release of perforin, which forms a pore in the target cell membrane. The perforin pore leads to osmotic imbalances (Persechini et al., 1990) and allows for entry of granzymes into the target cell inducing cell death (Trapani and Smyth, 2002). The second mechanism is mediated by the expression of Fas ligand and subsequent activation of the Fas death receptor pathway, leading to programmed cell death of the target cell (Kagi et al., 1994a; Kagi et al., 1994b; Lowin et al., 1994; Trapani and Smyth, 2002). In addition to direct cytotoxicity, CD8⁺ T cells also contribute to viral clearance through the release of proinflammatory cytokines, such as MIP-1alpha, IL-2, TNF-alpha, and IFN-gamma (Cocchi et al., 1995; Guidotti and Chisari, 1996). In particular, IFN-gamma plays a critical role in sensitizing target cells to cytolysis, enhancing antimicrobial activities of the innate immune system, and interfering with components of the viral life cycle (Schroder et al., 2004).

1.3.2 Generation of lifelong CD8⁺ T cell memory after acutely resolved infections

The population of terminal effector cells is short-lived and rapidly cleared after pathogen clearance (Joshi et al., 2007; Kaech et al., 2003; Murali-Krishna et al., 1998). A second population, however, persists after this contraction phase and matures into the

long-lived memory population that supports immunity in the case of pathogen re-exposure. A number of events have been suggested to influence the development of this memory precursor population, including exposure to inflammatory signals (Joshi et al., 2007), such as CpG (Pham et al., 2009), IL-2 (Manjunath et al., 2001; Pipkin et al., 2010), and IL-12 (Takemoto et al., 2006), as well as asymmetric cell division (Chang et al., 2007).

The events that regulate the evolution from memory precursors to long-lived memory cells, however, are less well understood. After the contraction phase, the initial surviving population of virus-specific cells has low expression of lymphoid homing markers (CD62L and CCR7) and resembles an effector memory T cell population (Wherry et al., 2003b). Over time, this population matures into a central memory pool that effectively homes to lymphoid organs and homeostatically self-renews (Wherry et al., 2003b). What signals regulate this maturation process is not well understood.

One pathway that is known to influence the rate of memory maturation is the mammalian target of rapamycin (mTOR) pathway. Low dose treatment with an inhibitor of the mTOR signaling cascade not only enhances the generation of memory precursor CD8⁺ T cells during the expansion phase, but also independently accelerates the memory precursor maturation towards central memory (Araki et al., 2009). The ability of rapamycin to enhance memory differentiation has been suggested to be due to changes in the expression of T-box transcription factors (discussed below) (Rao et al., 2010).

1.3.3 Maintenance of lifelong CD8⁺ T cell memory to acutely resolved infections

Once formed, memory T cells undergo slow homeostatic division in response to the homeostatic cytokines IL-7 and IL-15 (Becker et al., 2002; Goldrath et al., 2002; Tan

et al., 2002). This division is non-hierarchical as each long-lived memory cell has an equal probability of entering division (Choo et al., 2010) and has been suggested to predominantly occur in the bone marrow (Becker et al., 2005). In contrast, once memory cells encounter their cognate antigen, they proliferate and differentiate, giving rise to a population of short-lived cells that contribute to the anti-pathogen response (Ciocca et al., 2012; Dutton et al., 1998; Wherry et al., 2003b). At the same time, however, these activated memory cells renew the long-lived memory pool to ensure immunity in the event of future infection (Ciocca et al., 2012; Wherry et al., 2003b). In this way, memory T cells can effectively provide for lifelong protection against acutely resolved infections.

1.3.4 Responses to persistent antigen and inflammation: CD8⁺ T cell exhaustion

Chronic infections with high rates of pathogen replication as well as malignancy pose a different and perhaps more difficult challenge for maintaining anti-pathogen or anti-tumor responses. In contrast to acute infection, where the immune system returns to a state of quiescence after pathogen clearance, chronic infections and malignancy lead to sustained antigen and inflammation. How CD8⁺ T cells maintain such responses over years or decades has appeared paradoxical.

CD8⁺ T cell responses to chronic viral infection in mice and humans have been characterized by their poor proliferative responses to *in vitro* antigenic stimulation (Migueles et al., 2002; Wherry et al., 2004; Zajac et al., 1998; Brenchley, 2003 #1353). Thus, it was possible that a prolonged antiviral (or anti-tumor) response was maintained by long-lived “exhausted” cells that have low rates of proliferation and cell turnover. Other investigators, however, have observed that exhausted CD8⁺ T cells have elevated rates of proliferation and cell turnover. For example, CD8⁺ T cells from chronically infected humans and mice display increased rates of apoptosis (Blackburn et al., 2010;

Gougeon et al., 1996; Lewis et al., 1994; Meyaard et al., 1992) which are matched by an extensive proliferative history (Brenchley et al., 2003), likely a result of high rates of cell turnover and *in vivo* proliferation (Hellerstein et al., 1999; Hellerstein et al., 2003; McCune et al., 2000; Sachsenberg et al., 1998; Shin et al., 2007). How to reconcile these discordant findings has remained unclear. **Chapter 3** of this thesis will provide data to define the population dynamics of exhausted CD8⁺ T cell responses.

A second feature of CD8⁺ T cell exhaustion is a reduction in effector functionality, such as cytotoxic potential and proinflammatory cytokine expression (Wherry, 2011). This dysfunction is associated with the upregulation of a variety of surface molecules, termed inhibitory receptors, which limit cytotoxic lymphocyte responses. Higher expression of a single inhibitory receptor (Blackburn et al., 2010; Day et al., 2006; Nakamoto et al., 2008) or enhanced co-expression of multiple of inhibitory receptors (Blackburn et al., 2009; Jin et al., 2010; Matsuzaki et al., 2010; McMahan et al., 2010; Nakamoto et al., 2009; Sakuishi et al., 2010) correlates with reduced CD8⁺ T cell effector function. In addition, blockade of a single (Barber et al., 2006; Brahmer et al., 2012; Day et al., 2006; Nakamoto et al., 2008; Raziorrouh et al., 2010; Topalian et al., 2012; Velu et al., 2009) or a combination (Blackburn et al., 2009; Jin et al., 2010; Matsuzaki et al., 2010; McMahan et al., 2010; Nakamoto et al., 2009; Sakuishi et al., 2010; Zhou et al., 2011) of inhibitory receptors restores CD8⁺ T cell function and leads to improved viral or tumor control.

In addition to the expression of inhibitory receptors, immunoregulatory cytokines have also been implicated in the development of viral persistence and CD8⁺ T cell exhaustion. In particular, deletion or blockade of IL-10 (Brooks et al., 2008; Brooks et al., 2006) or TGF-beta (Tinoco et al., 2009) has demonstrated these cytokines impede viral

elimination. Along with restoration of effector functions, most therapeutic interventions are associated with improved proliferation and expansion of antiviral or anti-tumor CD8⁺ T cell responses. Thus, whether the beneficial effects of therapeutic intervention are a result of enhanced quality of CD8⁺ T cell effector functions, expansion of the total CD8⁺ T cell population, or a combination thereof remains to be determined. **Chapter 3** will provide a framework to understand how such interventions might influence the population dynamics of the CD8⁺ T cell response.

1.4 T-box transcription factors

Specification of differential cellular function and behavior is programmed through a network of transcriptional regulators. The family of T-box transcription factors, named after the founding member Brachyury (T), contributes to this specification during a diverse set of developmental processes, including trophoblast, limb, brain, heart, and immune cell differentiation (Naiche et al., 2005). The T-box family is defined by a conserved DNA binding domain that has been suggested to prefer the DNA consensus sequence TCACACCT (Naiche et al., 2005).

1.4.1 Activity of T-box transcription factors is context-dependent

T-box transcription factors regulate their target genes through a variety of mechanisms. T-box factors appear to be capable of both activation and repression of target genes. This differential activity appears to be dependent, in part, on other nuclear factors, such as histone methyltransferases (Lewis et al., 2007), suggesting that T-box factors may operate in a context-dependent manner. In support of this hypothesis, introduction of transcriptional repressors belonging to the Ripply or BTB-POZ family converts the activating activity of Tbx24 and T-bet (Tbx21) into repressive activity, respectively (Kawamura et al., 2008; Oestreich et al., 2011; Oestreich et al., 2012).

Furthermore, despite the apparent conserved binding affinity to the DNA consensus sequence and equivalent recruitment of histone methyltransferases by multiple family members, T-box factors still maintain cell-specific activity (Lewis et al., 2007). Thus, T-box factors may be highly dependent on the co-expressed transcriptional network in order to appropriately regulate target genes.

Oppositional activity of two related T-box family members was first hypothesized to occur between Tbx4 and Tbx5 in limb development. In the developing embryo, Tbx4 and Tbx5 are expressed in the hindlimb and forelimb, respectively. Mutations in Tbx4 lead to a human syndrome with lower limb deformities (Bongers et al., 2004), while mutations in Tbx5 lead to Holt-Oram syndrome with upper limb malformations (Basson et al., 1997; Li et al., 1997), suggesting that differential T-box factor expression conferred differential limb fate. Consistent with this hypothesis, inappropriate ectopic expression of Tbx4 or Tbx5 led to the development of hindlimbs or forelimbs, respectively, or features thereof (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Thus, two T-box factors may initiate and maintain distinct transcriptional programs within similar or analogous cell types.

1.4.2 T-box factors regulate self-renewal and differentiation

While T-box transcription factors have a variety of roles during embryogenesis and development, several family members also regulate tissue homeostasis in the adult organ. For example, Tbx1 regulates the renewal of long-lived progenitors in the hair follicle. Deletion of Tbx1 leads to an accelerated decline of these follicle stem cells after repeated regenerative stress (Chen et al., 2012). Other T-box family members regulate the development of terminally differentiated cells. For example, Eomesdermin (Eomes) supports the amplification and differentiation of neurons in the embryonic subventricular

zone (Arnold et al., 2008) and the adult dentate gyrus (Hodge et al., 2012). Thus, T-box factors can support either long-lived, quiescent progenitors or short-lived, terminally differentiated progeny.

1.4.3 T-box factors regulate antiviral immunity

The two T-box factors that are expressed in immune cells are T-bet and Eomesodermin (Eomes). These two factors belong to the same T-box subfamily, which they share with T brain 1 (Tbr1) (Szabo et al., 2000). T-bet and Eomes share 74% identity in the T-box domain, but only 25% identity in the flanking C-terminal and N-terminal regions (Pearce et al., 2003). While T-bet is expressed in a variety of immune cells associated with an antiviral Type 1 response (Lugo-Villarino et al., 2003; Sullivan et al., 2003; Szabo et al., 2000; Szabo et al., 2002; Townsend et al., 2004; Wang et al., 2012), Eomes expression is largely restricted to cytotoxic lymphocytes (Pearce et al., 2003) and potentially CD4⁺ T cells engaged in a Type 1 response (Qui et al., 2011; Suto et al., 2006; Yagi et al., 2010; Yang et al., 2008). One exception, however, may be a specific subpopulation of T_H2 CD4⁺ T cells that express IL-5 (Endo et al., 2011).

1.4.4 T-bet and Eomes in Natural Killer cells

Natural killer (NK) lymphocyte development is initiated in the bone marrow with the generation of the NK precursor (NKp). Further progression through development to an intermediate stage is marked by the upregulation of NK cell antigens such as NK1.1 and NKp46 as well as the death ligand TRAIL. While developmental arrest can occur at this stage, full NK cell maturation is associated with a proliferative burst and battery of phenotypic changes, including loss of TRAIL expression, upregulation of the inhibitory/activating Ly49 receptor family, induction of the integrins CD11b and CD49b,

and downmodulation of the costimulatory molecule CD27 (Gordon et al., 2012; Yokoyama et al., 2004).

T-bet and Eomes are both expressed during this process; however, the exact role these factors play in NK cell development has recently been illuminated. In the absence of Eomes, NK cells arrest at the intermediate stage of differentiation, displaying expression of TRAIL with minimal CD49b expression. Eomes is also necessary for maintaining the stable program of fully mature NK cells, since temporal deletion of Eomes in CD49b⁺ NK cells leads to the loss of CD49b expression and the reacquisition of TRAIL expression (Gordon et al., 2012). Thus, Eomes plays a critical role specifically in this second stage of NK maturation.

Unlike Eomes, T-bet appears to regulate earlier developmental steps. In the absence of T-bet, NK cells appear to have an accelerated developmental progression and lack cells that arrest at the intermediate TRAIL⁺ stage. In fact, temporal deletion of T-bet in intermediate NK cells leads to induction of Eomes expression and progression to a mature stage. Without either T-box factor, however, NK development does not progress past the NKp stage (Gordon et al., 2012). Thus, T-bet and Eomes appear to provide sequential specification during NK cell development.

1.4.5 T-bet and Eomes in CD8⁺ T cells during acute infection

In response to an acutely resolved infection, CD8⁺ T cells generate two populations of antiviral cells: short-lived, terminally differentiated effectors and long-lived, self-renewing memory. T-bet and Eomes appear to regulate the function, formation, and survival of both these populations. During the initial expansion after antigenic stimulation, T-bet and Eomes redundantly support cytotoxic effector programming

(Intlekofer et al., 2008; Zhu et al., 2010). This is likely a result of direct binding and transactivation of genes encoding cytotoxic molecules, such as perforin and granzyme B, and inflammatory cytokines, such as interferon gamma (Intlekofer et al., 2005 Cruz-Guilloty, 2009 #807; Pearce et al., 2003; Pipkin et al., 2010; Townsend et al., 2004). Loss of both transcription factors leads to ineffective antiviral responses and aberrant differentiation to a Type 17 response (Intlekofer et al., 2008). T-bet and Eomes also redundantly support CD8⁺ T cell longevity through positive regulation of the beta chain of the IL-15 receptor, CD122. In particular, Eomes directly binds to the CD122 locus and activates gene expression (Intlekofer et al., 2005).

T-bet and Eomes, however, also have opposing or alternative roles in CD8⁺ T cell responses to acutely resolved infection. Formation of terminally differentiated effector cells is dependent on the activity of T-bet (Intlekofer et al., 2007; Joshi et al., 2007) but unaffected by the loss of Eomes (Banerjee et al., 2010). The expression of T-bet during this process is regulated by environmental cues, such as CD4⁺ T cell help (Intlekofer et al., 2007), IL-12 signaling (Takemoto et al., 2006), and potentially other inflammatory signals (Joshi et al., 2007). The enhancement of the terminally differentiated population occurs at the expense of the memory precursor population (Intlekofer et al., 2007; Joshi et al., 2007; Takemoto et al., 2006).

In contrast, Eomes supports the development or maintenance of long-lived, self-renewing memory. Eomes expression is enriched in the memory pool after several months post viral clearance (Banerjee et al., 2010; Intlekofer et al., 2005) and genetic deletion of Eomes leads to a gradual decline in the memory population (Banerjee et al., 2010). Eomes deficient memory cells also display poor expansion after antigenic challenge (Banerjee et al., 2010). The impaired maintenance is thought to be secondary

to poor homing to the bone marrow for homeostatic proliferation. Thus, T-bet and Eomes act redundantly to initiate effector programming, yet support alternative CD8⁺ T cell fates. **Chapter 2** of this thesis will extend our understanding of when and how Eomes supports and directs the differentiation CD8⁺ T cells in response to acute viral infection.

1.4.6 T-bet in CD8⁺ T cells during chronic infection

The role for T-box factors in chronic viral infection is quite distinct from that of acutely resolved infection. Based on T-bet's essential role in the formation of terminal effectors during the initial expansion, one might predict that T-bet expression would remain elevated as CD8⁺ T cells mount a prolonged antiviral response. After contraction of the short-lived population, however, T-bet expression in both mice (Kao et al., 2011) and humans (Hersperger et al., 2011; Ribeiro-Dos-Santos et al., 2012) is down-regulated during chronic infection. The reduced expression of T-bet correlates with CD8⁺ T cell dysfunction, including lower perforin expression (Hersperger et al., 2011; Ribeiro-Dos-Santos et al., 2012), poor viral control (Hersperger et al., 2011), and higher inhibitory receptor expression (Kao et al., 2011). Furthermore, genetic reduction in T-bet expression leads to enhanced inhibitory receptor expression, reduced cytokine expression, and an inability to sustain antiviral CD8⁺ T cell responses (Kao et al., 2011). Thus, higher T-bet expression is associated with more effective antiviral responses to chronic infections. **Chapter 3** in this thesis will extend our understanding of the manner in which T-bet sustains antiviral responses during chronic viral infection.

1.4.7 Eomes in CD8⁺ T cells during chronic infection

Based on Eomes' regulation of effective cytotoxic effector programming and maintenance of long-lived, protective memory, one could imagine that dysfunctional responses to chronic viral infections would be associated with lower Eomes expression.

Microarray analysis, however, has suggested that Eomes is elevated in exhausted CD8⁺ T cells (Wherry et al., 2007). **Chapter 3** in this thesis will investigate the role for Eomes in this setting.

1.5 The Lymphocytic Choriomeningitis Model of Viral Infection

Lymphocytic Choriomeningitis Virus (LCMV) is a robust model of viral infection. Identified in 1934 by Charles Armstrong (Beeman, 2007), LCMV is a negative sense, single stranded RNA virus belonging to the Arenaviridae family. Viral entry is mediated by GP1 protein of LCMV binding to alpha-dystroglycan on host cells, allowing for multi-organ dissemination (Borrow and Oldstone, 1992; Cao et al., 1998). Clearance of viral infection requires an effective CD8⁺ T cell response (Jamieson et al., 1987; Moskophidis et al., 1987). Defects in CD8⁺ T cell effector functions have been demonstrated to impair viral clearance. For example, loss of IFN-gamma, Fas receptor activation, or perforin prevents effective viral control in LCMV-infected mice (Klavinskis et al., 1989; Leist et al., 1989; Nansen et al., 1999; Rode et al., 2004; Wille et al., 1989). As a result, LCMV provides an effective model to test CD8⁺ T cell mediated viral control.

1.5.1 Acutely resolved LCMV infection

The initial strain of LCMV, named Armstrong after its discoverer Charles Armstrong (Beeman, 2007), initiates an acutely resolved infection. In adult mice, this virus replicates predominantly in the spleen, liver, and kidney and is normally cleared within 10 days with no manifestation of disease (Wherry et al., 2003a). LCMV Armstrong elicits potent adaptive immune responses that provide effective protection to rechallenge. **Chapters 2 & 3** will use LCMV Armstrong to model CD8⁺ T cell responses to an acutely resolved viral infection.

1.5.2 Chronic LCMV infection

In contrast to infection of adult mice, introduction of LCMV Armstrong to neonatal mice leads to the development of a carrier state. These mice develop poor adaptive immune responses and are persistently infected with LCMV. A variant of LCMV Armstrong (clone 13) was isolated from the spleen of a carrier mouse. This variant was found to have acquired the capacity to persist in the adult host and establish a chronic infection (Ahmed et al., 1984). The ability of LCMV clone 13 to persist in the adult host was a result of two specific point mutations: one in the viral polymerase and one in the viral glycoprotein (Matloubian et al., 1993; Matloubian et al., 1990; Salvato et al., 1991; Sullivan et al., 2011). The mutation in the viral polymerase increases the polymerase activity and accelerates viral replication. The mutation in the viral glycoprotein increases the affinity of the viral glycoprotein for its receptor alpha-dystroglycan and broadens the tropism of the virus. Together, these two mutations are sufficient to convert LCMV from an acutely resolved infection to a chronic infection (Sullivan et al., 2011). Infection of immunocompetent mice with LCMV clone 13 leads to systemic viral replication with viremia lasting 2-3 months and lifelong viral reservoirs in the kidney and the brain (Wherry et al., 2003a). One benefit of the comparison between Armstrong and clone 13 is that the adaptive immune recognition between these two variants is identical. **Chapter 3** of this thesis will employ this comparison to investigate transcriptional differences that arise due to viral persistence. Furthermore, **Chapter 3** will use LCMV clone 13 as a robust model to investigate CD8⁺ T cell responses to viral infection.

CHAPTER 2: Fluorescent reporter reveals insights into Eomesodermin biology in cytotoxic lymphocytes

2.1 Introduction:

Cytotoxic lymphocytes play important roles in anti-viral and anti-tumor immune responses. Two major effector functions of NK and CD8⁺ T cells are the release of pro-inflammatory cytokines and the killing of virally infected or transformed target cells via the perforin and granzyme pathway. These pathways are regulated by several transcription factors, including Eomesodermin (Eomes). However, Eomes has also been reported to regulate other essential features of cytotoxic cells. As a result, the relative importance of Eomes activity on effector functions versus differentiation remains obscure.

Eomes supports a variety of differentiation events, ranging from mesoderm formation in the developing embryo (Russ et al., 2000) to lymphocyte differentiation in response to infection (Hertoghs et al., 2010; Intlekofer et al., 2008; Intlekofer et al., 2005). In the hematopoietic system, Eomes has been reported to direct the differentiation of multiple lymphocyte subsets. For example, Eomes plays an important role in formation and maintenance of mature TRAIL⁺DX5⁺ NK cells (Gordon et al., 2012; Intlekofer et al., 2005; Tayade et al., 2005). In CD8⁺ T cells, Eomes has pleiotropic roles, supporting the developmental program of both effector (Intlekofer et al., 2008) and memory (Banerjee et al., 2010; Intlekofer et al., 2005) T cells.

Eomes was identified in cytotoxic lymphocytes owing to its role in regulating expression of several effector molecules, e.g. IFN- γ , perforin, and granzyme B (Pearce

et al., 2003). Correspondingly, Eomes expression has been associated with enhanced CD8⁺ T cell cytotoxicity (Gao et al., 2005; Hegel et al., 2009; Hinrichs et al., 2008; Intlekofer et al., 2005; Pipkin et al., 2010; Rao et al., 2010; Tao et al., 2007) and anti-viral (Intlekofer et al., 2008) and anti-tumor (Atreya et al., 2007; Gao et al., 2005; Hinrichs et al., 2008; Rao et al., 2010; Tao et al., 2007; Zhu et al., 2010) responses. Eomes expression has also been reported in cytotoxic CD4⁺ T cells and is essential for expression of granzyme B in these cells (Qui et al., 2011). Thus, Eomes is thought to be integral to the program of cytotoxic gene expression in multiple lymphocyte lineages and may provide an opportunity for therapeutic targeting.

Consequently, CD8⁺ T cells that are induced or programmed to express high levels of Eomes would be predicted to have increased expression of effector molecules and enhanced lytic potential. Indeed, Eomes expression is heterogeneous within CD8⁺ T cell populations, displaying a large dynamic range (Banerjee et al., 2010; Gordon et al., 2011). Whether CD8⁺ T cells with high Eomes expression produce more cytotoxic molecules and therefore exhibit higher cytolytic capacity than Eomes non-expressers has not been examined.

Eomes expression is also particularly important for the maintenance of central memory CD8⁺ T cells (Banerjee et al., 2010), in part through its transcriptional regulation of the gene encoding IL-15R β (CD122), which enhances IL-15 responsiveness (Intlekofer et al., 2005). However, whether expression of Eomes during the expansion phase of the CD8⁺ T cell response is associated with improved formation or survival of central memory cells has not been determined. The initial Eomes reporter mouse required the addition of substrate in order to generate transient fluorescence (Intlekofer et al., 2005; Russ et al., 2000). A subsequent fluorescent protein-based construct with

spontaneous fluorescence grossly correlated with *Eomes* expression in the developing embryo (Kwon and Hadjantonakis, 2007), yet displayed incomplete fidelity in the developing brain (Arnold et al., 2009; Kwon and Hadjantonakis, 2007). A novel *Eomes* fluorescent reporter mouse, however, where GFP is expressed from the *Eomes* locus in lieu of *Eomes* more effectively paralleled *Eomes* expression (Arnold et al., 2009). Here, we report the validation and use of this *Eomes*^{gfp} targeted allele in the study of *Eomes* gene expression in NK and CD8⁺ T cells.

In CD8⁺ T cells, we were able to separate *Eomes* expressors (GFP⁺) from *Eomes* non-expressors (GFP⁻) by flow cytometry and address specific cytotoxic capacity of these subsets. Unexpectedly, *Eomes* expression was not associated with enhanced lytic potential in effector CD8⁺ T cells; however, early *Eomes* expression did correlate with improved central memory formation. Furthermore, examination of *Eomes*^{gfp} expression in the absence of *Eomes* protein suggested that *Eomes*⁺ central memory CD8⁺ T cells may be dependent on *Eomes* expression for persistence. Lastly, reporter activity in *Eomes*-deficient NK cells allowed for the identification of putative intermediates in NK cell development that are primed for full maturation into TRAIL⁺DX5⁺ NK cells. Thus, the *Eomes*^{gfp} targeted allele should provide a novel opportunity to further understand the role of *Eomes* in cytotoxic lymphocytes.

2.2 Methods

2.2.1 Mice and Infection:

All animals were housed at the University of Pennsylvania (Philadelphia, PA). Experiments were performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. *Eomes*^{gfp/+} mice have been

described previously (Arnold et al., 2009). To study Eomes GFP reporter activity during Fas deficiency, Eomes^{gfp/+} mice were mated to Fas^{lpr/lpr} mice. To study Eomes GFP reporter activity in the absence of Eomes protein, Eomes^{gfp/+} mice were mated to Eomes^{flox/flox} mice with or without CD4-Cre or Vav-Cre. To study Eomes^{gfp} expression during anti-viral responses, mice were infected with 2×10^5 PFU of lymphocytic choriomenigitis virus (LCMV) Armstrong strain by i.p. injection. For some experiments, Eomes^{gfp/+} mice were also mated to P14 TCR-Tg mice to generate P14 Eomes^{gfp/+} mice. 5×10^4 Eomes^{gfp/+} P14 CD8⁺ T cells (CD90.1) were transferred to WT B6 mice (CD90.2) 1 day prior to infection with LCMV Armstrong.

2.2.2 Flow cytometry, division dye labeling, and real-time PCR:

Surface and intracellular staining were performed as described previously (Gordon et al., 2011; Intlekofer et al., 2008). Antibodies used for flow cytometry were purchased from BD Biosciences (CD3, CD4, CD8, CD19, CD27, CD44, CD62L, CD122, DX5, NK1.1; San Jose, CA) or eBioscience (CD45.1, CD45.2, CD90.1, CD107a, CD127, Eomes, KLRG1, TRAIL; San Diego, CA). Intracellular staining for GFP was performed with polyclonal anti-GFP (eBioscience) followed by goat anti-rabbit Alexa Fluor 488 secondary (Invitrogen, Carlsbad, CA). Data were collected on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). Cell sorting was performed using a BD Aria II (BD Biosciences). For indicated experiments, sorted cells were subsequently labeled with CellTrackerTM Violet (Invitrogen) according to manufacturer's instructions. qRT-PCR was carried out as previously described (Intlekofer et al., 2008). Target gene probes were purchased from Applied Biosystems (Foster City, CA).

2.2.3 Cytotoxicity assay:

Splenocytes from wild-type CD45.1 mice were labeled with two different concentrations (50 nM = “dim” or 2 μ M = “bright”) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen). CFSE^{dim} target splenocytes were incubated with LCMV-derived NP396-404 peptide, while CFSE^{bright} control splenocytes were incubated with ovalbumin-derived SIINFEKL peptide for 1h at 37°C. 5×10^3 target cells were mixed with 5×10^3 control cells. GFP⁺ and GFP⁻ CD8⁺ T cells from d8 LCMV-infected mice were separated by cell sorting and percentages of GFP⁺ and GFP⁻ NP396-specific CD8⁺ T cells were determined by flow cytometry after tetramer staining. Increasing doses of GFP⁺ and GFP⁻ NP396-specific CD8⁺ T cells were added to wells containing target and control cells. Unlabeled, wildtype CD45.2 CD57BL/6 splenocytes were used to normalize cell concentrations in each well. After 6 or 24 hr incubation at 37°C, target cell destruction was assessed by flow cytometry. % specific lysis was calculated as $100 * [1 - (\% \text{ target cell remaining} / \% \text{ control cell remaining})]$.

2.3 Results

2.3.1 Expression of Eomes^{gfp} is largely restricted to cytotoxic lineages and correlates with Eomes protein expression.

Eomes expression has been reported in a variety of cell types during different stages of lymphocyte differentiation. We first validated whether expression of Eomes^{gfp} faithfully matched Eomes expression by correlating GFP expression with previous reports of Eomes mRNA or protein.

In the hematopoietic system, Eomes is expressed predominantly in cytotoxic lymphocytes (Pearce et al., 2003). We therefore determined whether Eomes^{gfp} expression followed a similar distribution within leukocyte lineages. In Eomes^{gfp/+} mice, NK and CD8⁺ T cells contained the highest frequency of GFP⁺ cells, while GFP expression in CD4⁺ T cells, B cells, and other lymphocyte lineages remained low or undetectable (**Fig 2.1A**). The small population of GFP⁺ CD4⁺ T cells may represent a recently described subset of memory T_H2 cells (Endo et al., 2011). In other blood cells, such as monocyte and granulocyte populations, GFP expression was undetectable (data not shown).

As GFP expression was heterogeneous in NK and CD8⁺ T cells (**Fig 2.1A**), we examined whether Eomes^{gfp} activity matched previous reports of Eomes protein expression in different CD8⁺ T cell subsets. We have recently identified an important role for Eomes in the generation of central memory CD8⁺ T cells (Banerjee et al., 2010). Consistent with Eomes protein expression (Banerjee et al., 2010), central memory phenotype (CD44^{hi}CD62L^{hi}) CD8⁺ T cells (T_{cm}) from unmanipulated mice exhibited a high frequency of GFP expression, whereas naïve (CD44^{lo}) and effector memory phenotype (CD44^{hi}CD62L^{lo}) CD8⁺ T cells (T_{em}) had lower frequency of GFP expression (**Fig 2.1B**). Notably, a minority of central memory phenotype CD8⁺ T cells remained GFP⁻. We therefore hypothesized that Eomes might regulate a subset of central memory phenotype CD8⁺ T cells.

Figure 2.1

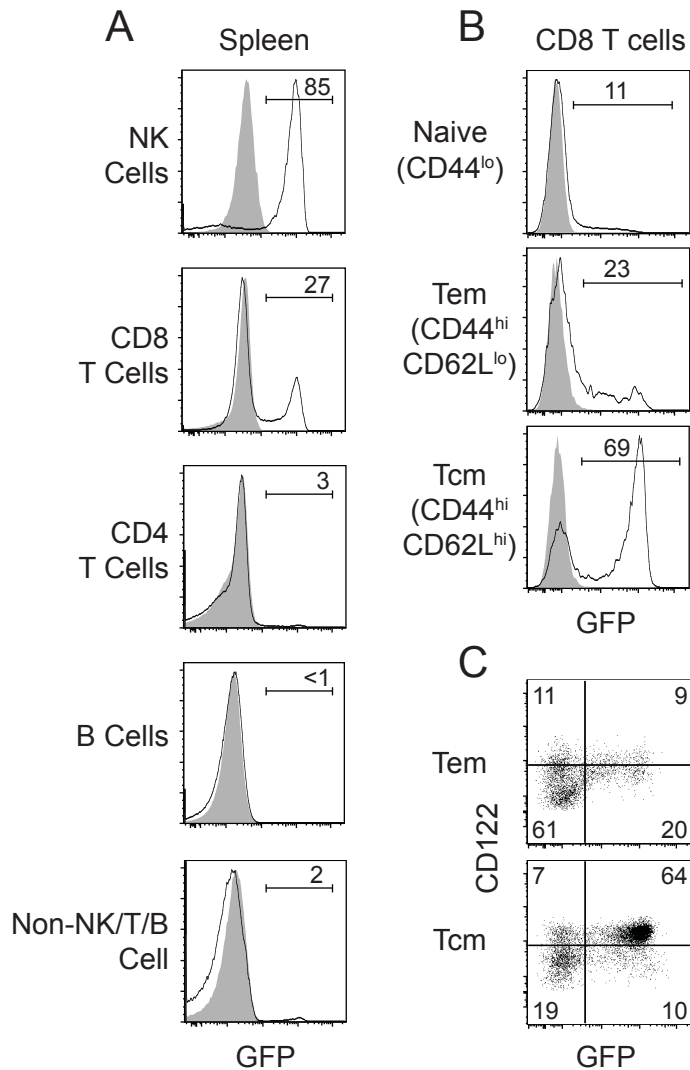


Figure 2.1. Expression of $Eomes^{gfp}$ is largely restricted to cytotoxic lineages and correlates with $Eomes$ protein expression.

Flow cytometry of GFP expression in indicated subpopulations of (A) mature splenocytes and (B) splenic CD8⁺ T cells from unmanipulated $Eomes^{+/+}$ (filled histograms) and $Eomes^{gfp/+}$ (open histograms) mice. (C) GFP expression positively correlates with a target of $Eomes$, CD122, in central memory CD8⁺ T cells. Flow cytometry of CD122 versus GFP expression in effector and central memory phenotype CD8⁺ T cells. Numbers denote frequency within the gated population.

The beta chain for the IL-15 receptor (CD122), a direct target of Eomes (Intlekofer et al., 2005), is important for homeostasis of memory CD8⁺ T cells by supporting basal homeostatic proliferation (Becker et al., 2002; Goldrath et al., 2002; Tan et al., 2002). We therefore examined whether Eomes^{gfp} expression correlated with a known Eomes transcriptional target in memory CD8⁺ T cells. Effector memory phenotype CD8⁺ T cells expressed low levels of both GFP and CD122. In contrast, central memory phenotype CD8⁺ T cells expressed high levels of CD122 and GFP, with a high frequency of GFP and CD122 co-expression (**Fig 2.1C**). Thus, the Eomes^{gfp} targeted allele matches Eomes protein expression and trans-activation by Eomes.

2.3.2 GFP expression quantitatively parallels Eomes protein expression.

We next addressed whether activity of the Eomes^{gfp} locus matched quantitative changes in Eomes protein expression. Mice and humans with mutations in Fas or FasL suffer from autoimmune and lymphoproliferative syndrome (ALPS). A major feature of ALPS is lymphadenopathy and splenomegaly due to the accumulation of αβ TCR-bearing CD4⁻CD8⁻ (double-negative [DN]) T cells. These DN T cells express substantial levels of Eomes and Eomes expression is critical for their accumulation (Kinjyo et al., 2010). We chose to employ this model of Fas deficiency to examine whether activity of the Eomes^{gfp} locus would quantitatively match enhanced Eomes protein expression. Examination of NK, CD4⁺, CD8⁺, and DN T cells from Fas^{WT/WT} Eomes^{gfp/+} mice revealed that GFP⁺ cells in each population expressed similar amounts of GFP per cell as measured by MFI of GFP⁺ cells (**Fig 2.2A**). In Fas^{lpr/lpr} Eomes^{gfp/+} mice, however, CD4⁺ and CD8⁺ T cells exhibited a 1.5 fold enhancement of GFP expression over NK cells, while DN T cells demonstrated greater than 2-fold increase in GFP expression (**Fig 2.2A**). The fold enrichment of GFP expression paralleled increases in Eomes protein

Figure 2.2

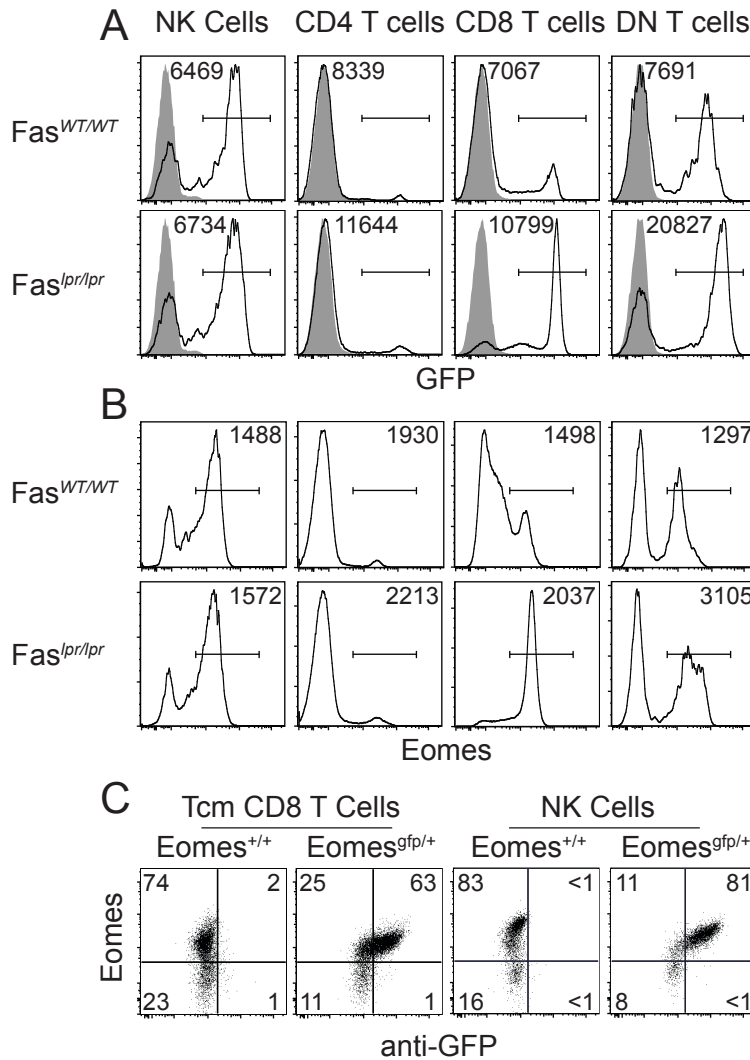


Figure 2.2. GFP expression quantitatively matches Eomes protein expression. Flow cytometry of (A) GFP or (B) Eomes protein expression in indicated splenic subpopulations from Eomes^{+/+} (filled histograms) and Eomes^{gfp/+} (open histograms) mice with indicated Fas genotype. Numbers denote GFP MFI and Eomes MFI of GFP⁺ and Eomes⁺ cells, respectively. (C) GFP expression is specific for Eomes expression in lymphocytes. Flow cytometry of intracellular staining for Eomes and GFP protein in NK and central memory CD8⁺ T cells from Eomes^{+/+} and Eomes^{gfp/+} mice.

expression (**Fig 2.2B**). Thus, the dynamic range of Eomes expression in DN T cells during ALPS is primarily regulated at the transcriptional level. In addition, the activity of the Eomes^{gfp} locus can discriminate between quantitative changes in Eomes protein levels.

To further confirm that Eomes^{gfp} expression correlates with Eomes protein expression in NK and CD8⁺ T cells, we directly compared Eomes and GFP protein levels. GFP fluorescence is lost upon fixation and permeabilization for Eomes protein staining due to direct quenching of fluorescence, loss of GFP protein from the cell, or a combination thereof. As a result, we measured GFP expression by directly staining for GFP protein using an anti-GFP antibody followed by an Alexa Fluor 488 conjugated secondary antibody. Intracellular staining revealed a positive correlation between Eomes and GFP protein expression, as all GFP⁺ cells were Eomes⁺ (**Fig 2.2C**). Of note, a fraction of both NK and CD8⁺ T cells expressed Eomes protein without immunologic detection of GFP protein (**Fig 2.2C**). Consequently, Eomes^{gfp} locus activity may be a specific, but not entirely sensitive, reporter of Eomes protein expression in lymphocyte populations.

2.3.3 Eomes expression does not identify the most cytotoxic effector CD8⁺ T cells.

Eomes has been reported to transcriptionally activate several genes associated with cytotoxic capacity, such as those encoding perforin and granzyme B protein (Pearce et al., 2003; Pipkin et al., 2010). Furthermore, *in vitro* culture conditions that induce heightened expression of Eomes in CD8⁺ T cells are associated with more potent cytotoxicity than those conditions resulting in lower Eomes (Pipkin et al., 2010). However, direct analysis of whether Eomes expression leads to enhanced cytotoxic potential is lacking. To this end, Eomes^{gfp/+} reporter mice were infected with lymphocytic

choriomeningitis virus (LCMV) Armstrong to assess whether Eomes expression correlates with enhanced cytotoxicity in effector CD8⁺ T cells.

We first examined whether Eomes^{gfp} expression enriched for terminal effector or memory precursor cells. We have previously reported that Eomes protein is equally distributed between these two subsets (Banerjee et al., 2010). Consistent with our previous observations, virus-specific GFP⁺ and GFP⁻ CD8⁺ T cells displayed equal expression of terminal effector markers such as KLRG1 and memory markers such as CD127 or CD27 (**Fig 2.3A**).

To determine whether Eomes expression conferred enhanced cytolytic potential, we assessed whether Eomes expression was associated with enhanced degranulation and increased expression of cytotoxic molecules. As peptide stimulation leads to TCR downregulation and loss of tetramer staining, we utilized cells from P14 TCR transgenic mice, where we could track virus-specific cells by congenic markers. Eomes^{gfp/+} P14 CD8⁺ T cells were isolated one week post-infection and stimulated with cognate peptide. GFP⁺ and GFP⁻ P14 CD8⁺ T cells displayed equal levels of degranulation as measured by CD107a (LAMP-1) staining (**Fig 2.3B**). We next evaluated whether Eomes expression led to enhanced transcription of cytotoxic molecules. Eomes^{gfp/+} P14 CD8⁺ T cells from LCMV-infected mice were sorted based on GFP expression. Comparison of GFP⁺ and GFP⁻ effector CD8⁺ T cells revealed a modest reduction in the expression of granzyme B and perforin mRNA in GFP⁺ cells compared to GFP⁻ cells, although this trend was not statistically significant (**Fig 2.3C**).

Figure 2.3

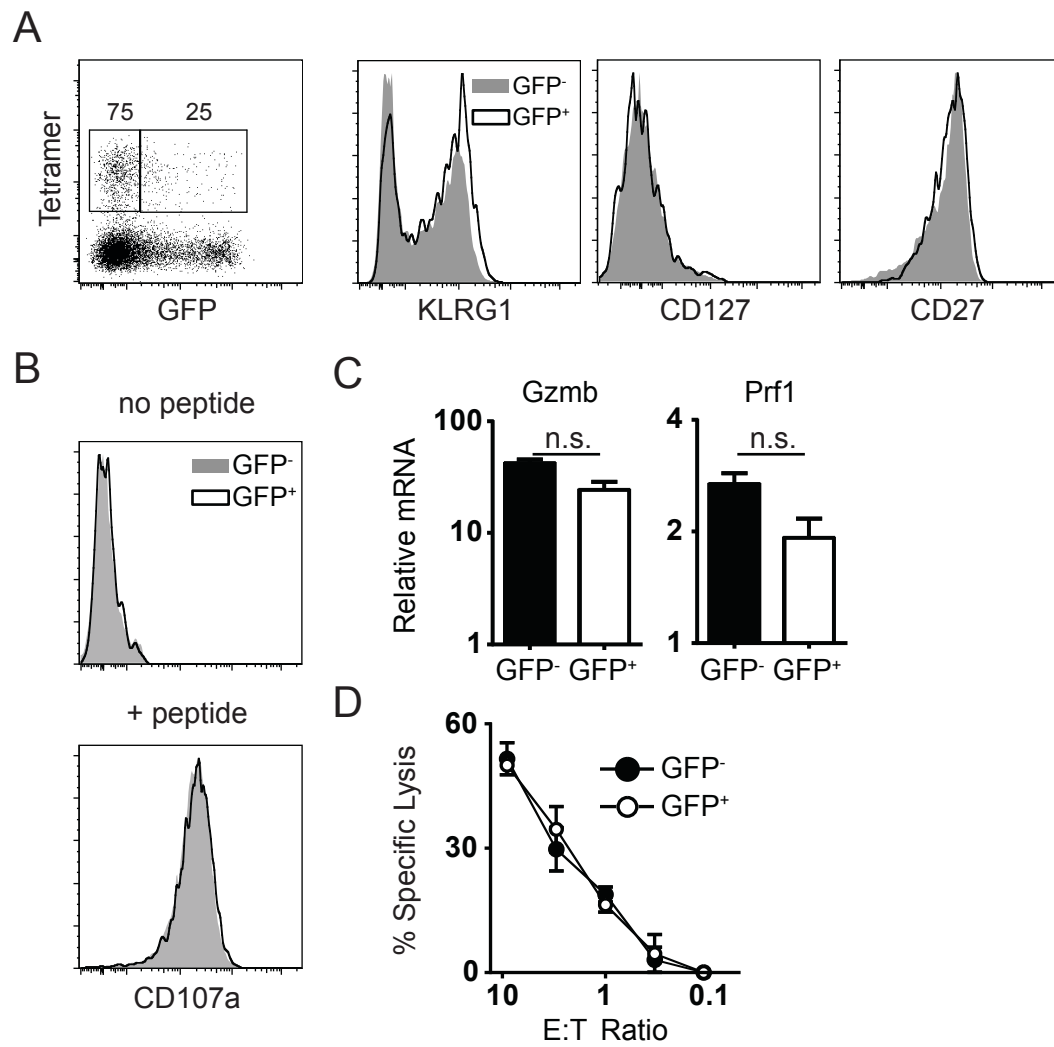


Figure 2.3. Eomes expression does not correlate with enhanced cytotoxicity.

Endogenous or P14 Eomes^{gfp/+} CD8⁺ T cell responses were assessed after LCMV infection. (A) GFP expression in CD8⁺ T cells is not biased towards terminal effector or memory precursor cells. GFP⁺ and GFP⁻ np396-specific CD8⁺ T cells were assessed for KLRG1, CD127, and CD27 expression 8 days post-infection. (B) Eomes expression does not correlate with enhanced degranulation. Eomes^{gfp/+} P14 CD8⁺ T cells were stimulated with or without D^bgp33-41 peptide 7 days post-infection. Degranulation was assessed by CD107a staining. (C) Eomes expression does not correlate with enhanced transcription of cytotoxic molecules. Eomes^{gfp/+} P14 CD8⁺ T cells were sorted based on GFP expression 7 days post-infection and assessed for Perforin and Granzyme B mRNA expression. mRNA levels were normalized to HPRT and are reported relative to naïve cells. (n.s.; Wilcoxon matched pairs test). (D) Cytolytic activity of GFP⁺ and GFP⁻ CD8⁺ T cells. Sorted GFP⁺ and GFP⁻ CD8⁺ T cells were equalized for NP396-specific CD8⁺ T cells and added to target cells pulsed with LCMV-derived peptide NP396-404. % specific lysis was determined by the loss of target cells compared to control cells. Graphs displays mean \pm S.E.M.

Last, we determined whether Eomes expression influenced the cytotoxic potential of effector CD8⁺ T cells. Sorted GFP⁺ and GFP⁻ CD8⁺ T cells displayed equivalent cytotoxicity on a per cell basis in an *in vitro* killing assay (**Fig 2.3D**). Thus, while Eomes may activate genes encoding cytotoxic molecules early after T cell activation *in vitro*, Eomes expression does not correlate with enhanced cytotoxicity in fully differentiated *in vivo* generated effector T cells. Consequently, robust killing may be independent of Eomes expression at this stage of the CD8⁺ T cell response.

2.3.4 Progressive enrichment of Eomes expression in memory CD8⁺ T cells.

Compared to effector CD8⁺ T cells at the peak of cellular expansion, memory CD8⁺ T cells have elevated Eomes expression (Banerjee et al., 2010; Intlekofer et al., 2005). Whether this elevated expression is due to selection of Eomes⁺ memory CD8⁺ T cells, gradual induction of Eomes expression in Eomes⁻ memory CD8⁺ T cells, or a combination of the two is unclear. To assess whether Eomes enrichment is due to selection or induction, we first examined GFP expression longitudinally in virus-specific Eomes^{gfp/+} CD8⁺ T cells after infection with LCMV.

As noted above, GFP expression was elicited in virus-specific CD8⁺ T cells at the peak of expansion (d8). During the contraction phase, however, a consistent reduction in GFP expression was observed (d8-d15) (**Fig 2.4A**). Nevertheless, as the memory population matured (d15-d60), GFP expression was progressively enriched (**Fig 2.4A**), consistent with elevated Eomes expression in late virus-specific memory (Banerjee et al., 2010; Intlekofer et al., 2005).

As Eomes expression is important for central memory CD8⁺ T cells (Banerjee et al., 2010), it is possible that expression of Eomes early after activation would lead to

enhanced memory formation (Rao et al., 2010), however this hypothesis has not been formally examined. To determine whether early Eomes expression correlates with the formation of long-lived, Eomes⁺ CD8⁺ T cells, P14 Eomes^{gfp/+} CD8⁺ T cells were sorted based on relative GFP expression 8 days post-infection and transferred into infection-matched controls. While analysis of GFP expression revealed a modest downregulation of Eomes in the first week after viral clearance, 80-90% of GFP⁺ cells retained Eomes expression (**Fig 2.4B, 2.5A**). In contrast, GFP⁻ cells largely remained GFP⁻ (**Fig 2.4B, 2.5A**). Thus, the majority of Eomes expression in the memory population appears to arise during the initial phase of the antiviral response.

We next tested whether early Eomes expression was associated with improved memory differentiation. P14 Eomes^{gfp/+} CD8⁺ T cells that were separated by GFP expression at day 8 post-infection gave rise to equal numbers of memory cells, with a modest trend towards enhanced homing to the bone marrow (**Fig 2.5B**). The CD8⁺ T cell population with early Eomes expression, however, did mature more rapidly to central memory, as measured by the expression of CD62L (**Fig 2.5C, D**). Thus, while early Eomes expression may not be associated with enhanced survival into the memory phase, early induction of the *Eomes* locus correlates with more efficient central memory formation.

2.3.5 Expression of the Eomes locus is reduced in the absence of Eomes protein.

We next evaluated whether CD8⁺ T cells stably transcribe the *Eomes* locus in the absence of Eomes protein. To test this, we generated Eomes^{gfp/flox} mice with or without Cre recombinase under the control of the CD4 promoter (CD4-Cre).

Figure 2.4

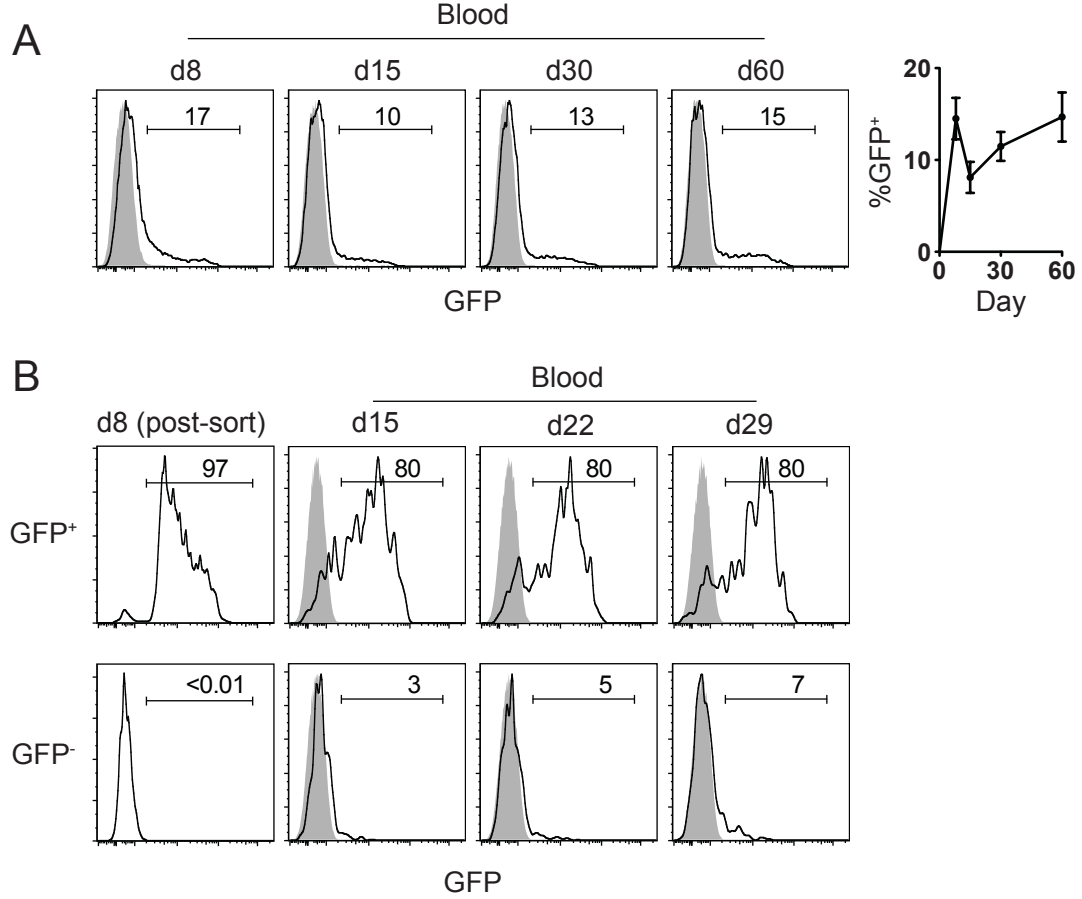


Figure 2.4. Progressive enrichment of Eomes expression in memory CD8⁺ T cells. Endogenous or P14 Eomes^{gfp/+} CD8⁺ T cell responses were assessed after LCMV infection. (A) Induction of Eomes expression is biphasic. Longitudinal GFP expression in gp33-specific CD8⁺ T cells from the blood of Eomes^{gfp/+} mice at indicated days post-infection. (B) Early expression of Eomes is predominantly stable. Eomes^{gfp/+} P14 CD8⁺ T cells were sorted based on GFP expression 8 days post-infection and transferred into infection-matched controls. Plots display GFP expression of sorted populations in the blood at indicated days post-infection. Numbers indicate frequencies within gated populations. Graph displays mean \pm S.E.M.

Figure 2.5

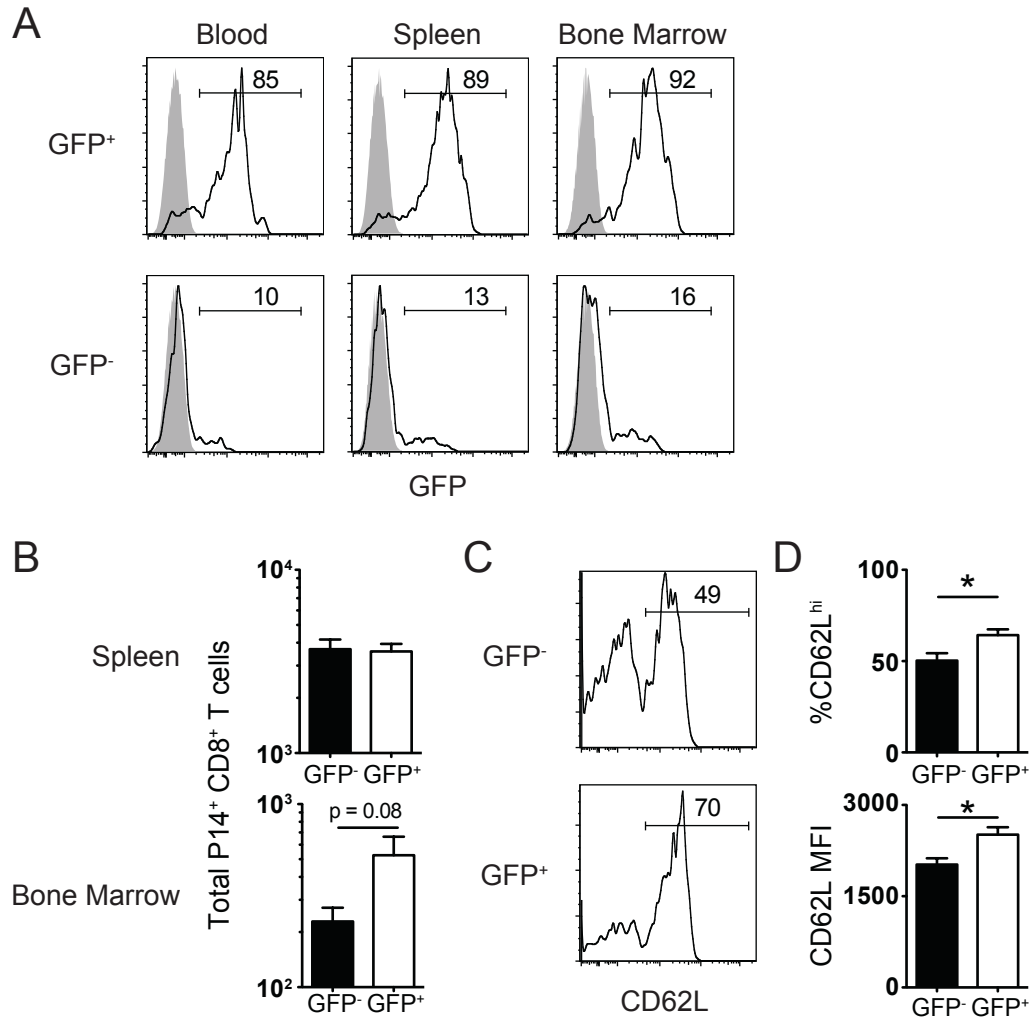


Figure 2.5. Early expression of Eomes is associated with more efficient central memory generation. Eomes^{gfp/+} P14 CD8⁺ T cells were sorted based on GFP expression 8 days post-infection and transferred into infection-matched controls as in **Fig 2.4B**. (A) Plots display GFP expression of sorted populations recovered from blood, spleen and bone marrow 45 days post-transfer (53 days post-infection). (B) Total P14 Eomes^{gfp/+} CD8⁺ T cells recovered in (A). (C) CD62L expression of sorted populations from (A). (D) Quantification of %CD62L^{hi} and CD62L MFI of CD62L^{hi} cells from (C). Numbers indicate frequencies within gated populations. Graphs display mean ± S.E.M.

Eomes^{gfp/flox} mice contain a high frequency of CD8⁺ T cells with a phenotype of long-lived, self-renewing central memory, e.g. expressing L-selectin (CD62L) and components of the IL-15R (CD122) and IL-7R (CD127) (**Fig 2.5; left column**). Consistent with a role for *Eomes* in the support of central memory CD8⁺ T cell differentiation (Banerjee et al., 2010), a majority of these central memory phenotype CD8⁺ T cells express GFP (**Fig 2.5; left column**). Deletion of *Eomes* led to reduced expression of all three markers of long-lived memory CD8⁺ T cells and to a reduced frequency of GFP⁺ cells (**Fig 2.5; right column**). In particular, less than of quarter of CD62L^{hi}, CD122^{hi}, or CD127^{hi} CD8⁺ T cells maintained GFP expression in the absence of *Eomes* protein (**Fig 2.5; right column**). Quantification of GFP⁺ and GFP⁻ central memory phenotype CD8⁺ T cells suggested that the lower GFP expression was due to specific loss of the *Eomes*⁺ subset rather than reduced *Eomes* locus activity (**Fig 2.5B**).

In the absence of *Eomes*, central memory CD8⁺ T cells fail to maintain a long-lived, stable population, presumably due to decreased homeostatic proliferation as a result of reduced bone marrow homing (Banerjee et al., 2010). To determine whether the specific loss of GFP⁺ central memory phenotype CD8⁺ T cells resulted from poor homeostatic proliferation, we sorted GFP⁺ CD44^{hi} CD62L^{hi} CD8⁺ T cells from *Eomes*^{gfp/flox} mice with or without CD4-Cre, labeled the cells with a cell proliferation dye (Violet Cell TrackerTM), and transferred them into congenically disparate hosts. Analysis of cell division after one month *in vivo* demonstrated reduced homeostatic proliferation in *Eomes*-deficient CD8⁺ T cells (**Fig 2.6A**). Thus, central memory CD8⁺ T cells that activate *Eomes* transcription also become dependent upon *Eomes* protein for persistence.

Figure 2.6

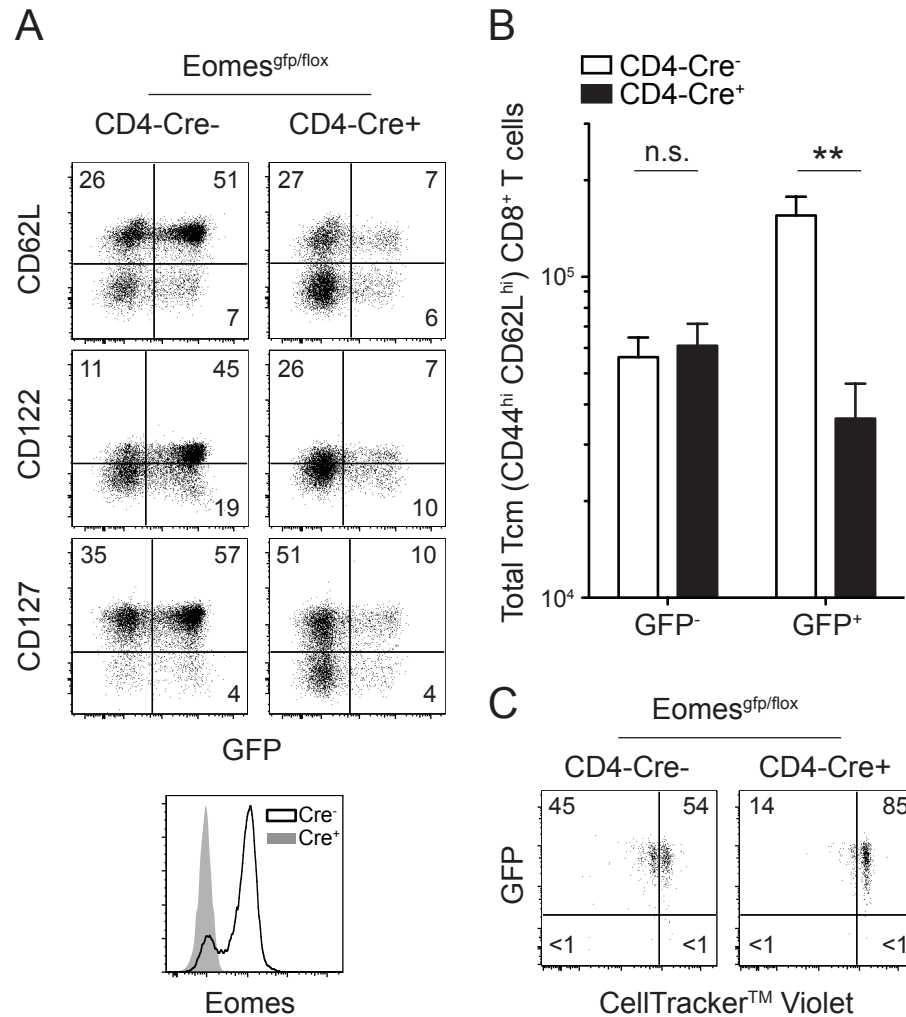


Figure 2.6. Central memory cells with an active *Eomes* locus are reduced in the absence of *Eomes* protein. (A) Flow cytometry of CD62L, CD122, and CD127 versus GFP expression in splenic *CD44^{hi} CD8⁺* T cells from mice with indicated genotype. Numbers denote frequency of cells in each quadrant. *Eomes* protein expression is also shown for the Cre-negative (open) and Cre-positive (filled) populations. (B) Total GFP⁻ and GFP⁺ central memory (*CD44^{hi} CD62L^{hi}*) cells in the presence (Cre-negative; open) or absence (Cre-positive; filled) of *Eomes* protein. (C) Sorted GFP⁺ central memory (*CD44^{hi} CD62L^{hi}*) cells were labeled with a cell division dye (CellTracker™ Violet) and transferred into *CD45.1* congenic recipients. Plots display GFP expression versus CellTracker™ Violet after 4 weeks *in vivo*.

2.3.6 Eomes^{gfp} allows for detection of putative intermediates of NK cell development.

TRAIL⁺DX5⁻ NK cells appear to represent developmental intermediates of NK cell maturation. Adoptive transfer of TRAIL⁺DX5⁻ NK cells has been shown to generate TRAIL⁻DX5⁺ NK cells in recipient mice (Gordon et al., 2012; Takeda et al., 2005). Deletion of Eomes, however, prevents NK cells from progressing past the TRAIL⁺DX5⁻ precursor stage (Gordon et al., 2012). Whether TRAIL⁺DX5⁻ NK cells activate Eomes expression prior to the maturation into TRAIL⁻DX5⁺ NK cells is not known. To investigate whether Eomes transcriptional activation occurs prior to maturation, we examined Eomes^{gfp} activity in NK cells in the presence or absence of Eomes protein.

We first determined that GFP expression correlated with markers of NK maturation. In Eomes^{gfp/+} mice, GFP expression was restricted to TRAIL⁻DX5⁺ NK cells (**Fig 2.7A**), consistent with Eomes protein expression (Gordon et al., 2012). In the absence of Cre, Eomes^{gfp/flox} NK cells are able to express one functional copy of Eomes and appropriately generate a high frequency population of TRAIL⁻DX5⁺ NK cells that express both GFP and Eomes protein (**Fig 2.7B,C; left column**). As expected, NK cells from Eomes^{gfp/flox} mice harboring Cre had a severe reduction in the frequency of mature TRAIL⁻DX5⁺ NK cells and a selective enrichment for TRAIL⁺DX5⁻ precursors (**Fig 2.7B,C; right column**). Nevertheless, Eomes deficient NK cells contained a small population of cells that expressed high levels of both GFP and TRAIL (**Fig 2.7B; top row**). The dual expression of GFP and TRAIL suggests that a population of TRAIL⁺DX5⁻ precursors receive differentiation signals and activate Eomes transcription in the initial stages of the development of mature NK cells.

Figure 2.7

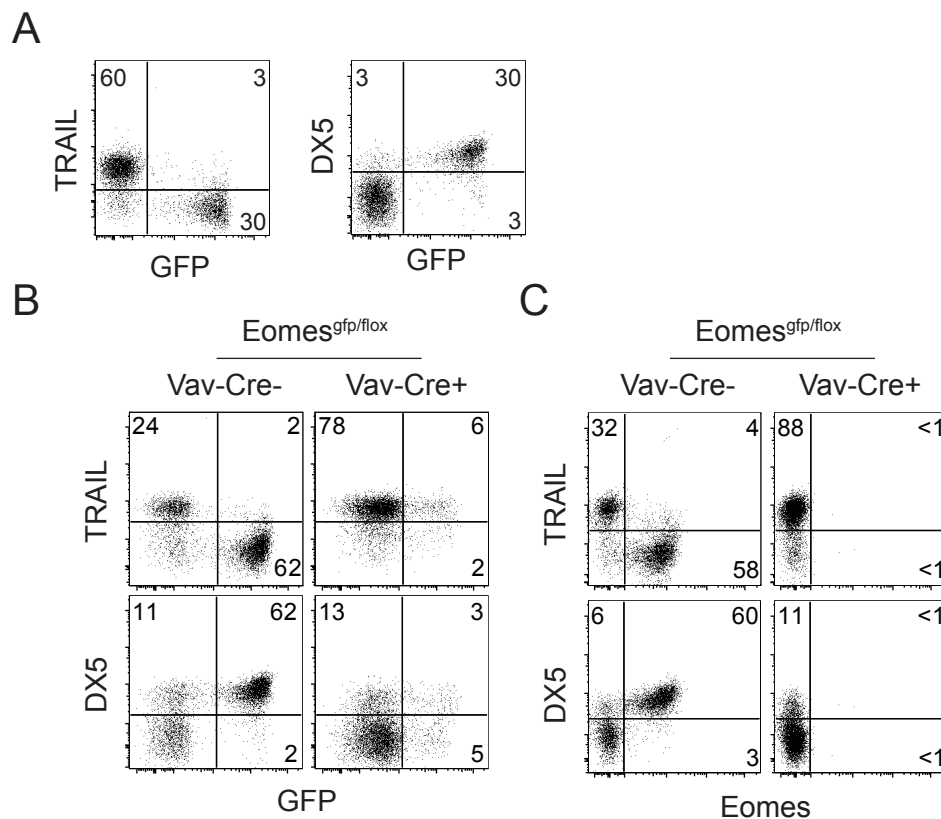


Figure 2.7. Eomes^{gfp} allows for detection of putative intermediates of NK cell development. (A) GFP is predominantly expressed in mature TRAIL⁻ DX5⁺ NK cells. Flow cytometry of TRAIL, DX5 and GFP expression in liver NK cells. (B) Flow cytometry of TRAIL or DX5 versus GFP or Eomes protein expression in liver NK cells from mice with indicated genotype. Numbers denote frequency of cells in each quadrant.

2.4 Discussion

In this study, we described the GFP expression of an *Eomes*^{gfp} targeted allele in the adult leukocyte compartment and matched *Eomes* and GFP expression in mature lymphocytes. While *Eomes* expression did not segregate with differential cytotoxicity in effector CD8⁺ T cells, early *Eomes* expression did lead to more efficient central memory formation. In addition, central memory CD8⁺ T cells that activate the *Eomes* locus may become dependent on *Eomes* protein for persistence and homeostatic proliferation. Lastly, GFP expression in *Eomes*-deficient NK cells enabled the identification of a putative intermediate of NK cell development.

Retroviral-based overexpression of *Eomes* appears to initiate a cytotoxic transcriptional program, activating effector genes such as IFN- γ , granzyme B, and perforin (Cruz-Guilloty et al., 2009; Pearce et al., 2003; Pipkin et al., 2010). Furthermore, in cultures with high IL-2 concentration, higher expression of *Eomes* correlates with CD8⁺ T cell killing capacity (Hinrichs et al., 2008; Pipkin et al., 2010). However, whether the enhanced killing capacity is a direct result of increased *Eomes* expression was not addressed. With the availability of an *Eomes*^{gfp} reporter, we were able to separate cells with high *Eomes* expression from cells with negative or low *Eomes* expression within the same population. We found that higher *Eomes* expression did not correlate with higher expression of the cytotoxic molecules granzyme B and perforin nor with enhanced cytotoxicity in fully differentiated effector CD8⁺ T cells. One possible resolution to this paradox is that *Eomes* may play a more important role earlier, in acquiring cytotoxic potential, rather than in exerting cytotoxic function. Alternatively, the equivalent cytotoxic molecule expression and cytotoxicity of GFP⁺ and GFP⁻ subsets may be due to the redundancy of other transcription factors at this stage of the CD8⁺ T cell response. In

particular, the T-box factor T-bet plays a redundant role with Eomes in the induction of the cytotoxic program in effector CD8⁺ T cells (Intlekofer et al., 2008). Indeed, previous work indicates that deletion of Eomes did not appreciably affect the killing capacity of effector CD8⁺ T cells (Intlekofer et al., 2008).

Other transcription factors may also contribute to cytotoxic gene expression in the absence of Eomes. Notch (Cho et al., 2009) and Runx3 (Cruz-Guilloty et al., 2009) can both bind to the granzyme B and perforin loci and activate their transcription. In addition, differential signaling through Akt can modulate perforin expression without altering T-bet or Eomes expression (Macintyre et al., 2011), suggesting T-box factor independent pathways for cytotoxic gene regulation. As a result, multiple transcription factors may compensate for lack of Eomes in activating cytotoxic genes in effector CD8⁺ T cells.

We have recently reported a role for Eomes in the generation of central memory CD8⁺ T cells in response to viral infection (Banerjee et al., 2010). As the homologous T-box factor T-bet drives the terminal differentiation of effector CD8⁺ T cells, one might hypothesize that Eomes would conversely drive formation of memory precursors. Eomes expression, however, did not correlate with markers of memory precursor cells, nor did deletion of Eomes impair the generation of memory precursors (Banerjee et al., 2010). Nevertheless, early Eomes expression was associated with more rapid accumulation of central memory CD8⁺ T cells. Therefore, Eomes likely supports memory differentiation after the stage of memory cell fate commitment. When Eomes begins to contribute to the program of memory differentiation, however, remains to be determined.

In CD8⁺ T cells expressing markers of long-lived, self-renewing central memory, Eomes protein expression was important for maintaining cells with stable expression of the *Eomes* locus. This failed persistence of CD8⁺ T cells that would normally express *Eomes* transcripts in the absence of Eomes protein appeared to be due to the impaired homeostatic proliferation. Thus, Eomes⁻ central memory CD8⁺ T cells may contain a distinct program for survival compared to Eomes⁺ central memory cells.

We and others have reported the development of mature TRAIL⁻DX5⁺ NK cells from TRAIL⁺DX5⁻ NK cells. However, the signals that initiate full maturation of TRAIL⁺DX5⁻ precursors are unknown. Furthermore, the transcriptional events initiating the developmental program of NK maturation are similarly obscure. By examining Eomes-deficient NK cells carrying an Eomes^{gfp} reporter allele, we were able to identify a small population of TRAIL⁺DX5⁻ precursors that had activated Eomes transcription and potentially initiated subsequent steps in NK cell maturation. However, in the absence of Eomes protein, these GFP⁺TRAIL⁺ cells did not accumulate. Future studies will be needed to determine whether these cells fail to persist in the absence of Eomes protein as they progress through NK cell development or whether they return to the TRAIL⁺DX5⁻ precursor program and repress Eomes expression. Further exploration of GFP⁺ TRAIL⁺DX5⁻ precursors offers a novel opportunity to define the factors influencing NK cell maturation.

Correlation of GFP protein with Eomes protein in Eomes^{gfp/+} mice revealed a population of NK and CD8⁺ T cells that express Eomes without GFP. The specificity, but incomplete sensitivity, of the inserted EGFP in the Eomes locus raises new potential insights into the regulation of Eomes expression in lymphocytes. One possibility is that the level of GFP protein needed for antibody or fluorescent detection is greater than the

level of Eomes protein required for equivalent detection. A second possibility, however, is that Eomes protein exhibits a longer half-life than that of EGFP. Thus, low transcriptional activity of the Eomes locus may lead to low levels of Eomes protein but undetectable levels of EGFP. This notion is consistent with the observation that small differences in Eomes mRNA (Intlekofer et al., 2005) can lead to large differences in Eomes protein (Banerjee et al., 2010). Investigations into the control of Eomes protein stability could allow for the potential to manipulate Eomes concentration in both healthy (Banerjee et al., 2010; Gordon et al., 2011; Gordon et al., 2012; Intlekofer et al., 2008; Intlekofer et al., 2005) and diseased states (Kinjyo et al., 2010).

The ability to separate lymphocytes based on graded transcription factor expression is restricted to either the identification of correlative surface markers (Joshi et al., 2007) or the generation of reporter mice. This need is especially compelling in the case of transcription factors that exhibit a large dynamic range of expression within a single population. Thus, the generation of an Eomes^{gfp/+} reporter that exhibits robust fidelity to Eomes protein expression in cytotoxic lymphocytes should prove to be a beneficial tool in elucidating how heterogeneous Eomes expression results in differential fate or function.

CHAPTER 3: A critical role for balancing progenitor and terminal subsets of exhausted CD8⁺ T cells during chronic viral infection

3.1 Introduction:

Chronic viral infections, such as HIV, HBV, and HCV, contribute to substantial morbidity and mortality worldwide (Virgin et al., 2009). Defects in adaptive immune responses, such as CD8⁺ T cell exhaustion, limit complete viral control (Wherry, 2011). Exhausted CD8⁺ T cells do, however, provide some level of containment of these viruses. For example, depletion studies in SIV infected macaques or HCV infected chimpanzees demonstrate the central importance of T cells in these settings (Grakoui et al., 2003; Jin et al., 1999; Schmitz et al., 1999; Shoukry et al., 2003). Moreover, molecular escape from CD8⁺ (and CD4⁺) T cell responses during chronic viral infection demonstrates the important pressure that even exhausted CD8⁺ T cell place on chronic viral infections. However, these antiviral CD8⁺ T cells are under tremendous pressure and often receive strong, ongoing antigenic stimulation throughout the course of chronic infection.

Previous examination of T cells during HIV infection revealed a dramatic increase in the level of CD8⁺ T cell proliferation and turnover during the chronic phase of infection (Hazenberg et al., 2000; Hellerstein et al., 1999; McCune et al., 2000; Sachsenberg et al., 1998). One contributing factor to this high rate of CD8⁺ T cell turnover may be the virus-specific CD8⁺ T cell response. Consistent with this hypothesis, in a murine model of chronic viral infection, virus-specific CD8⁺ T cells displayed extensive proliferation *in vivo* (Shin et al., 2007). The high rate of replication and cell turnover, however, may also lead to detrimental effects on the CD8⁺ T cell response. For example, HIV-infected

individuals with higher rates of T cell proliferation and corresponding immune activation have an accelerated rate of disease progression (Giorgi et al., 1999; Hazenberg et al., 2000). Thus, how virus-specific CD8⁺ T cells might adapt during chronic infections to continuously repopulate the anti-viral CD8⁺ T cell pool over a prolonged period are unknown.

After initial infection and effector expansion, CD8⁺ T cells undergo contraction both when the infection is rapidly cleared and when the pathogen persists. Following acute infections, memory CD8⁺ T cell populations form. These memory CD8⁺ T cells return to quiescence but acquire the ability to self-renew in an antigen-independent manner, utilizing IL-7 and IL-15 for their maintenance (Becker et al., 2002; Goldrath et al., 2002; Lau et al., 1994; Murali-Krishna et al., 1999; Tan et al., 2002; Wiehagen et al., 2010). During chronic infection, however, antigen-independent memory CD8⁺ T cells often do not develop. Instead, a stable population of CD8⁺ T cells persists via a distinct, antigen-dependent type of maintenance that remains poorly understood.

Over the lifespan of an organism, several tissues experience extensive cell turnover and repeatedly repopulate a terminally differentiated population. These tissues, such as the epithelium of the skin and gut, adapt to these demands by having a collection of long-lived precursor cells that give rise to a short-lived and terminally-differentiated cell population. Often, this process is coupled to extensive division, allowing for a small population of progenitors to maintain the much larger population of cells that execute tissue-specific functions.

In the case of chronic infection, where virus-specific CD8⁺ T cells experience the stress of constant antigenic stimulation, a similar phenomenon might occur. In this case,

a small population of cells would be dedicated to longevity of the CD8⁺ T cell response by preserving the unique TCR sequence that confers antigen specificity. This small population, however, would act as precursors and would repeatedly give rise to a much larger, more differentiated, anti-viral CD8⁺ T cell population. Whether this process occurs in virus-specific CD8⁺ T cells during a chronic infection is unclear. Furthermore, what transcription factors would govern this process is not known.

Eomesodermin (Eomes) and T-bet are homologous T-box transcription factors (Pearce et al., 2003; Russ et al., 2000; Szabo et al., 2000) that are involved in a variety of developmental processes. Both transcription factors support the anti-viral CD8⁺ T cell response, in part through their activation of a number of genes encoding effector molecules, such as IFN- γ , perforin, and granzyme B (Intlekofer et al., 2008; Lewis et al., 2007; Pearce et al., 2003; Szabo et al., 2000; Townsend et al., 2004). Consequently, expression of these factors has been associated heretofore with effective and functional CD8⁺ T cell responses.

In some cases, however, two related T-box factors may promote alternative cell states along similar differentiation processes. For example, in acutely-resolved infections, T-bet directs CD8⁺ T cells to adopt a terminally-differentiated cell fate during the acute effector phase of the response (Intlekofer et al., 2007; Joshi et al., 2007), while Eomes supports the persistence of long-lived, self-renewing central memory CD8⁺ T cells long after the pathogen is cleared (Banerjee et al., 2010). Whether Eomes and T-bet govern alternative cell states in CD8⁺ T cells during a chronic viral infection, however, is unknown.

Here we report that, unlike T-bet which is down-regulated during dysfunctional CD8⁺ T cell responses to a chronic viral infection (Kao et al., 2011), Eomes is strongly upregulated in virus-specific CD8⁺ T cells. In addition, Eomes expression correlates with features of severe exhaustion. Nevertheless, Eomes expression is essential for maintaining the maximal CD8⁺ T cell response to limit viral replication. Eomes and T-bet support the persistence of two distinct subsets during the CD8⁺ T cell response to a chronic viral infection. T-bet^{hi} Eomes^{lo} CD8⁺ T cells exhibit low levels of proliferation and cell turnover *in vivo*; however these cells acts as progenitor cells that give rise to the T-bet^{lo} Eomes^{hi} population, a process that is coupled to extensive cell division. This amplified population then has a more limited ability to give rise to more progeny. Thus, Eomes and T-bet govern the balance between terminal differentiation and population renewal in CD8⁺ T cells during chronic viral infection.

3.2 Methods:

3.2.1 Human Subjects:

All human subjects were recruited at the Massachusetts General Hospital Gastrointestinal Unit and the Department of Surgery in accordance with the IRB approved study: "Cell mediated immunity in Hepatitis C virus infection"; Protocol # 1999-P-004983/54; MGH Legacy #: 90-7246. Liver specimens were obtained from explantation or resection of 8 patients with at least 5 years of chronic HCV infection, defined by positive anti-HCV antibody and detectable viral load. Three patients with treatment induced sustained virological response (SVR, undetectable viral load 6 months after end of treatment) were analyzed. SVR of all 3 patients was achieved at least 3 years prior to liver sampling. Intrahepatic lymphocytes (IHL) were extracted by

mechanical disruption of liver tissue. The cell suspension was then washed twice in RPMI 1640 culture medium supplemented with 10% FBS, followed by centrifugation at 300 rpm each time to remove cell debris and hepatocytes. The final washing step was followed by centrifugation at 1500 rpm to obtain a pellet of IHL.

3.2.2 Mice and Infections:

All animals were housed at the University of Pennsylvania (Philadelphia, PA). Experiments were performed in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. *Eomes*^{flox/flox}, *Eomes*^{gfp/+}, and *Tbx21*^{-/-} (T-bet KO) mice have been described previously (Arnold et al., 2009; Finotto et al., 2002; Intlekofer et al., 2008). To examine CD8⁺ T cell differentiation in the absence of *Eomes*, *Eomes*^{flox/flox} mice were crossed to CD4-Cre mice (*Eomes* cKO) (Intlekofer et al., 2008). CD4-Cre⁺ *Eomes*^{+/+} or littermate CD4-Cre⁻ *Eomes*^{ff} mice were used as WT controls. Mice were infected with either 2 x 10⁵ PFU of lymphocytic choriomeningitis virus (LCMV) Armstrong strain by i.p. injection to generate an acutely resolved infection or 4 x 10⁶ PFU of LCMV clone 13 strain by i.v. injection to generate a chronic infection. The V35A variant of clone 13 has been previously described (Blattman et al., 2009; Shin et al., 2007).

3.2.3 Flow cytometry and real-time PCR:

Surface staining was performed as described previously (Gordon et al., 2011; Intlekofer et al., 2008). Intracellular staining was performed using the Foxp3 / Transcription Factor Staining Buffer Set per manufacturer's instructions (eBioscience; San Diego, CA). Antibodies used for flow cytometry were purchased from BD Biosciences (CD4, CD8, CD19, CD44, 2B4, Ki-67; San Jose, CA), Biolegend (PD-1, Tim3, TNF- α , IFN- γ , T-bet; San Diego, CA), R&D Systems (MIP-1 α ; Minneapolis, MN),

or eBioscience (CD8, Lag3, CD160, 2B4, CD45.1, CD45.2, CD107a, Eomes). MHC class I peptide tetramers were made and used as described previously (Wherry et al., 2004; Wherry et al., 2003a). Data were collected on a BD LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). Cell sorting was performed using a BD Aria II (BD Biosciences). qRT-PCR was carried out as previously described (Gordon et al., 2012; Intlekofer et al., 2008). Gene expression was normalized to GAPDH. Target gene probes were purchased from Applied Biosystems (Foster City, CA).

3.2.4 Bone Marrow Chimeras

Bone marrow (BM) from WT (CD45.1⁺), T-bet KO (CD45.2⁺), and Eomes cKO (CD45.2⁺) donors was harvested. 5-10 x 10⁶ donor cells were transferred i.v. into sublethally irradiated (450 rads) *Rag2*^{-/-} recipients. 8 weeks after BM transfer, chimeras were infected with LCMV clone 13.

3.2.5 BrdU Treatment and Detection

Animals were treated with 2mg of BrdU (Sigma-Aldrich; St. Louis, MO) i.p. daily for 7 days prior to tissue harvest and analysis. BrdU incorporation was assessed by the BrdU Flow Kit per manufacturer's instructions (BD Biosciences).

3.2.6 CFSE labeling, temporal deletion and adoptive transfers:

At indicated days p.i., spleens were harvested from indicated CD45.2⁺ donor mice. CD8⁺ T cells were purified from splenocytes using magnetic beads according to manufacturer's protocol (CD8⁺ negative selection; MACS beads; Miltenyi Biotec, Auburn, CA). Purified CD8⁺ T cells were labeled with CFSE as described previously (Wherry et al., 2004; Wherry et al., 2003a), and transferred i.v. into CD45.1⁺ infection-matched

controls. Temporal Eomes deletion with Tat-Cre was performed as described (Gordon et al., 2012).

3.2.7 Statistical Analysis:

Student's t test (Paired and Unpaired), Mann-Whitney test, and 2-way ANOVA were performed using Prism software (Graphpad, La Jolla, CA).

3.3 Results:

3.3.1 Eomes expression is enhanced during chronic viral infection.

During chronic viral infections in humans and mice, T-bet expression is reduced in virus-specific CD8⁺ T cells and this reduction correlates with T cell dysfunction (Hersperger et al., 2011; Kao et al., 2011; Ribeiro-Dos-Santos et al., 2012). In contrast, microarray analysis of CD8⁺ T cells has suggested that Eomes mRNA is elevated during chronic infection (Wherry et al., 2007). We first validated these findings by RT-PCR in sorted virus-specific CD8⁺ T cells from an acutely resolved infection with lymphocytic choriomeningitis virus (LCMV) Armstrong (Arm) at day 8 (effector) and day 30 post-infection (memory) or from a chronic infection with LCMV clone-13 (cl-13) at day 30 post-infection (exhausted). Eomes expression was compared to naïve (CD44^{lo}) CD8⁺ T cells from uninfected mice. Consistent with microarray analysis, Eomes expression was strongly upregulated during chronic infection (**Fig 3.1A**). We next determined whether the elevated levels of Eomes was due to an increase in the frequency of cells expressing Eomes or an increase in Eomes expression per cell. Infection of an Eomes GFP reporter mouse (Eomes^{gfp/+}) (Arnold et al., 2009) with LCMV Arm generated a modest frequency

Figure 3.1

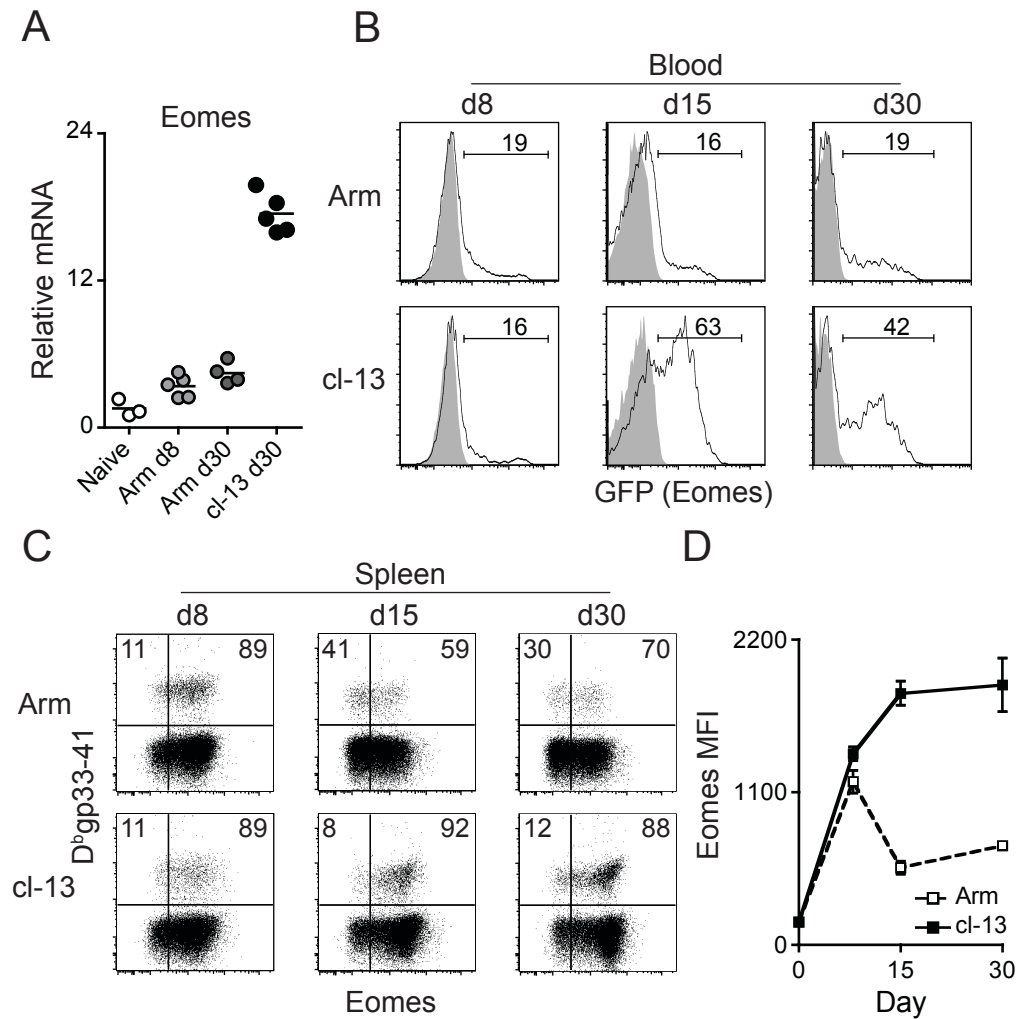


Figure 3.1. Eomes expression is enhanced during chronic viral infection. (A) Naïve or gp276-specific CD8⁺ T cells from effector (Arm d8), memory (Arm d30), or exhausted (cl-13 d30) time-points were sorted and analyzed for Eomes mRNA expression normalized to GAPDH. (B) Longitudinal histograms of GFP expression in gp33-specific CD8⁺ T cells from the blood of WT (shaded) or Eomes^{gfp/+} (open) mice at indicated days post Arm or cl-13 infection. (C) Eomes protein expression in gp33-specific CD8⁺ T cells from the spleens of WT mice 8, 15, or 30 days post-infection with Arm or cl-13. (B-C) Numbers indicate frequencies of gated populations. (D) MFI of Eomes expression from cells in (C).

of GFP⁺ virus-specific CD8⁺ T cells (**Fig 3.1B**). In contrast, infection of Eomes^{gfp/+} mice with LCMV cl-13 resulted in a robust frequency of GFP⁺ virus-specific CD8⁺ T cells (**Fig 3.1B**). Thus, the enhanced Eomes expression results – at least in part – from an increase in the frequency of cells expressing Eomes mRNA.

We next validated that the enhanced Eomes mRNA expression resulted in increased Eomes protein. Isolation of virus-specific CD8⁺ T cells from LCMV Arm or cl-13 infected mice revealed high levels of Eomes protein expression at the peak of the CD8⁺ T cell response (day 8 post-infection). Clearance of viral antigen during acute infection led to an initial drop in Eomes expression at day 15 that subsequently increased as the population began to mature into central memory (day 30). In contrast, high levels of Eomes protein expression persisted during chronic viral infection (**Fig 3.1C,D**). Thus, Eomes expression is highly upregulated in virus-specific CD8⁺ T cells during chronic viral infection.

3.3.2 Eomes expression correlates with features of CD8⁺ T cell exhaustion.

As CD8⁺ T cells during chronic viral infections exhibit heterogeneity in surface phenotype, transcriptional profile, and function, we examined how Eomes expression correlated with known features of CD8⁺ T cell exhaustion. We first investigated whether Eomes expression was correlated with the expression of inhibitory receptors, which have been demonstrated to impair optimal CD8⁺ T cell function during chronic viral infection (Barber et al., 2006; Blackburn et al., 2010; Blackburn et al., 2009; Wherry, 2011). Examination of virus-specific CD8⁺ T cells 30 days post-infection with LCMV cl-13 revealed a positive correlation between the expression of Eomes and several inhibitory receptors (PD-1, Lag3, CD160, & Tim3) (**Fig 3.2A**).

As Eomes appeared to correlate with markers of dysfunction (Blackburn et al., 2009), we next determined how Eomes expression related to other transcription factors that regulate CD8⁺ T cell responses during chronic viral infection. In particular, based on the positive correlation between Eomes and inhibitory receptor expression, we predicted a negative correlation between T-bet and Eomes (Kao et al., 2011) and a positive correlation between Eomes and Blimp1 (Shin et al., 2009).

After an acute infection with LCMV Arm, Eomes did not strongly correlate – positively or negatively – with the expression of T-bet (**Fig 3.2B; top row**). In contrast, virus-specific CD8⁺ T cells from chronically infected mice developed a prominent inverse correlation in the expression of T-bet and Eomes (**Fig 3.2B; bottom row**). In order to evaluate Blimp1 and Eomes co-expression, we crossed Blimp1 YFP reporter mice (Rutishauser et al., 2009; Shin et al., 2009) to Eomes^{gfp/+} mice. Dual reporter mice revealed a modest frequency of YFP and GFP co-expression at the peak of the CD8⁺ T cell response 8 days post-infection with LCMV Arm. This co-expression gradually declined as the cell-population matured towards central memory (**Fig 3.2C; top row**). Similar to acute infection, CD8⁺ T cells displayed a low frequency of YFP and GFP co-expression 8 days after LCMV cl-13 infection. However, chronic viral infection lead to a substantial frequency of co-expression of YFP and GFP that persisted for at least two months post-infection (**Fig 3.2C; bottom row**). Thus during chronic viral infection, Eomes expression correlates with CD8⁺ T cells that exhibit phenotypic and transcriptional characteristics of CD8⁺ T cell exhaustion.

Figure 3.2

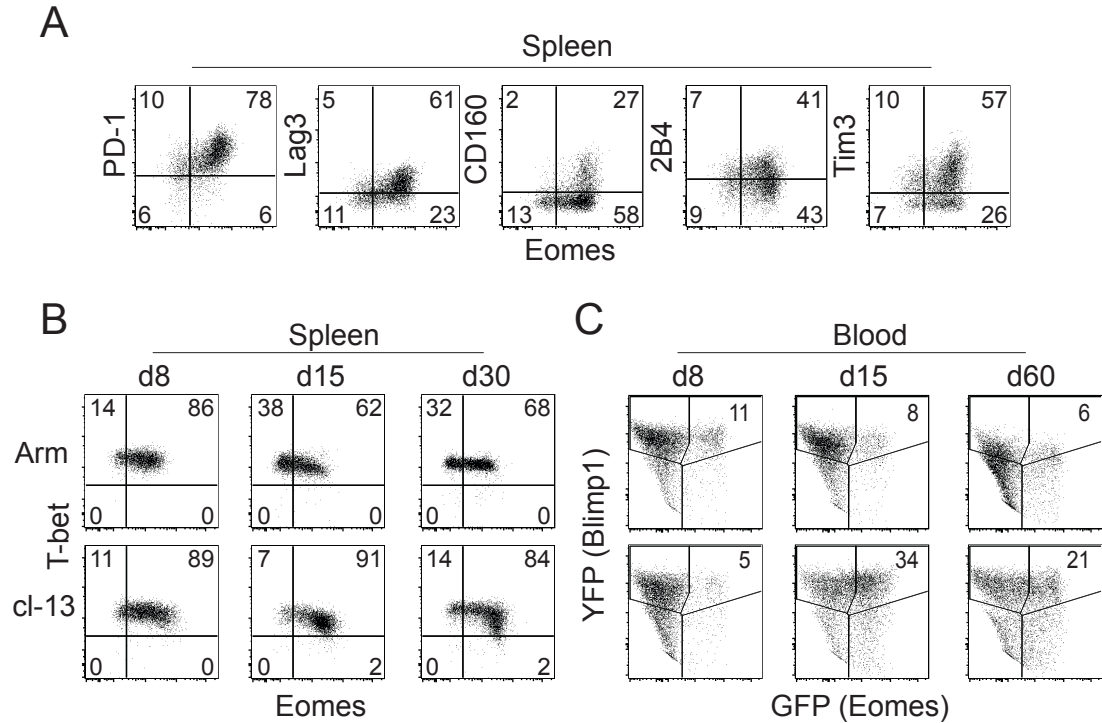


Figure 3.2. Eomes expression correlates with phenotypic and transcriptional features of CD8⁺ T cell exhaustion. (A) Eomes positively correlates with inhibitory receptor expression. Plots display indicated inhibitory receptor expression versus Eomes expression in gp33-specific CD8⁺ T cells 30 days post-infection. (B) T-bet and Eomes inversely correlate in virus-specific CD8⁺ T cells during chronic infection. T-bet versus Eomes expression in gp33-specific CD8⁺ T cells 8, 15, or 30 days post-infection with Arm or cl-13. Gates are based on naïve cells from uninfected mice. (C) Blimp1 and Eomes co-expression is enriched during chronic infection. Longitudinal frequency of GFP⁺YFP⁺ gp33-specific CD8⁺ T cells in the blood of Blimp1-YFP/Eomes^{gfp/+} dual reporter mice after Arm or cl-13 infection. Gates are based on WT and single reporter mice. Numbers denote frequency of gated population.

Figure 3.3

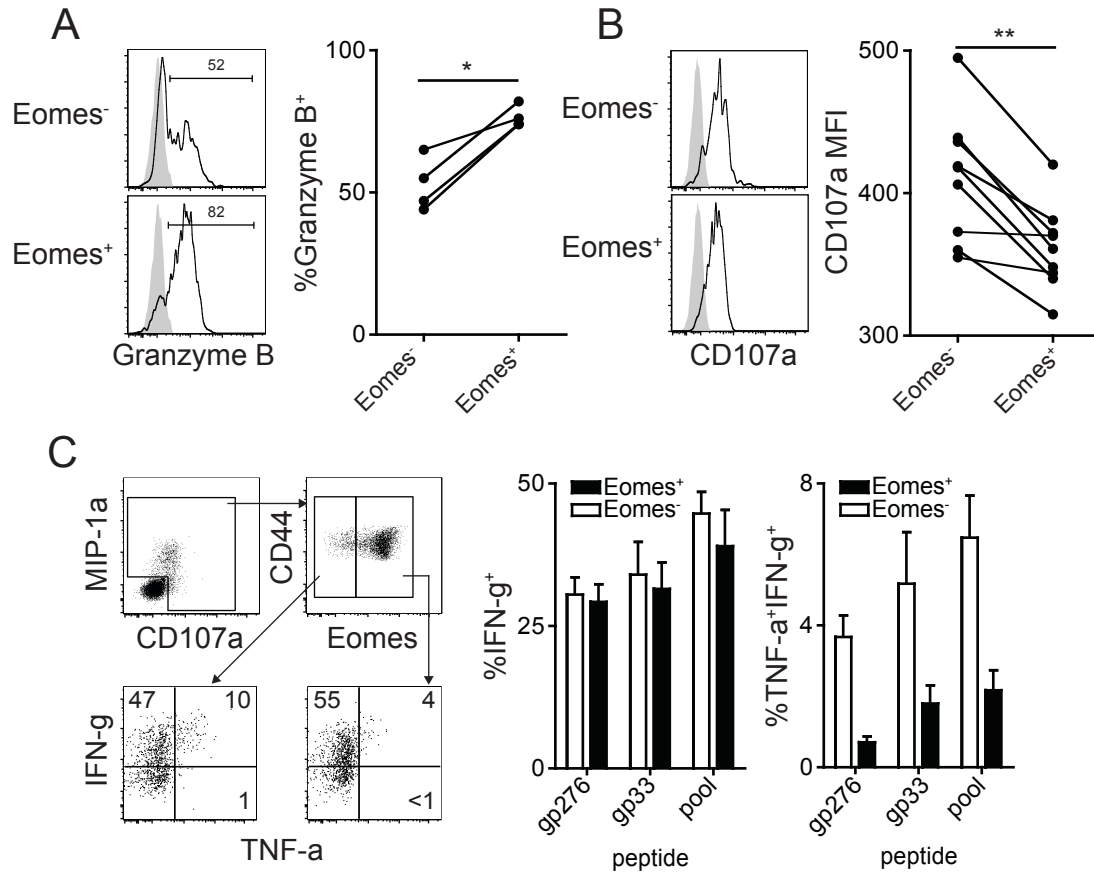


Figure 3.3. Eomes expression correlates with functional features of CD8⁺ T cell exhaustion. (A) Eomes expression correlates with increased granzyme B expression. Histograms of granzyme B expression in naïve (gray) or Eomes⁺ and Eomes⁻ gp276-specific CD8⁺ T cells (open) 22 days post-infection. (B) Eomes expression correlates with reduced degranulation. Histograms of CD107a staining of naïve (gray) or Eomes⁺ and Eomes⁻ MIP-1α⁺ CD8⁺ T cells (open) after stimulation with gp276-286 peptide on day 23 post-infection. (C) Eomes expression correlates with reduced co-expression of IFN-γ and TNF-α. 22 days post-infection, CD8⁺ T cells were stimulated with gp33-41, gp276-286, or a LCMV peptide pool and expression of IFN-γ and TNF-α was assessed for Eomes⁺ and Eomes⁻ CD8⁺ T cells. Numbers denote frequency of gated population.

We therefore examined how Eomes expression was associated with functional changes associated with CD8⁺ T cell exhaustion. We have previously reported an inverse correlation between inhibitory receptor expression and CD8⁺ T cell function during chronic infection (Blackburn et al., 2010; Blackburn et al., 2009). The impaired cytotoxicity is associated with reduced degranulation as measured by CD107a staining, yet paradoxically increased staining for granzyme B (Blackburn et al., 2010). Consistent with the positive correlation between Eomes and PD-1, granzyme B expression was enhanced in Eomes-expressing CD8⁺ T cells (**Fig 3.3A**), whereas CD107a staining was reduced (**Fig 3.3B**).

We have also reported that the expression of multiple inhibitory receptors inversely correlates with cytokine expression, in particular co-expression of IFN- γ and TNF- α (Blackburn et al., 2009). Therefore, we investigated whether Eomes expression correlated with differential cytokine expression. Examination of Eomes⁺ and Eomes⁻ CD8⁺ T cells that express MIP-1 α or stain for CD107a after peptide stimulation revealed equivalent frequencies of IFN- γ expression. In contrast, the ability to co-express IFN- γ with TNF- α was reduced in Eomes⁺ CD8⁺ T cells compared to Eomes⁻ CD8⁺ T cells (**Fig 3.3C**). Thus, Eomes expression is elevated in virus-specific CD8⁺ T cells that phenotypically, transcriptionally, and functionally display features of terminal exhaustion.

3.3.3 Deletion of Eomes leads to reduced features of CD8⁺ T cell exhaustion.

While WT CD8⁺ T cells had bimodal expression of T-bet and Eomes, genetic deletion of T-bet resulted in an increase in Eomes and PD-1 expression (**Fig 3.4A**), consistent with enhanced exhaustion (Kao et al., 2011). In contrast, Eomes-deficient CD8⁺ T cells had reduced PD-1 and Blimp1, as well as increased T-bet and functionality (**Fig 3.4B,C**).

Figure 3.4

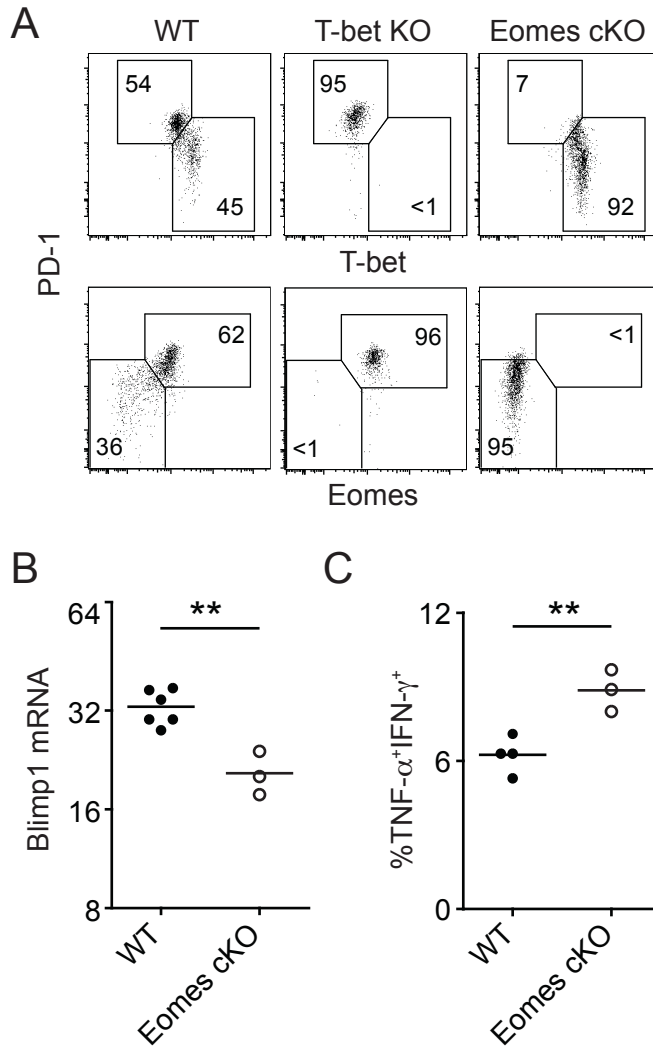


Figure 3.4. Eomes deficient CD8⁺ T cells appear less exhausted. (A) T-bet, Eomes, and PD-1 expression of D^bgp33-specific CD8⁺ T cells from WT, T-bet KO, and Eomes cKO mice on d22 p.i. Numbers denote frequency of gated population. (B) Blimp1 relative mRNA in D^bgp276-specific CD8⁺ T cells from WT and Eomes cKO mice on d15 p.i. (*p<0.05; Mann Whitney) (C) CD8⁺ T cells from WT and EKO mice were assessed for IFN-γ and TNF-α expression after peptide pool stimulation as in **Fig 3.3** on d22 p.i. (**p<0.01; Unpaired t test).

Figure 3.5

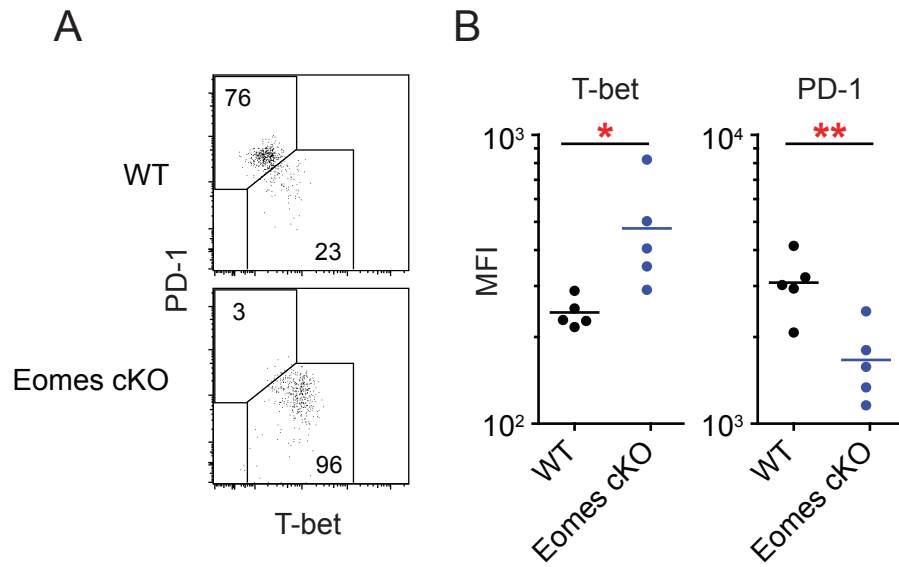


Figure 3.5. Eomes acts cell-intrinsically to maintain the more exhausted CD8⁺ T cell population. Rag KO hosts were sublethally irradiated and reconstituted with a 1:1 mixture of WT and Eomes cKO bone marrow, followed by infection with LCMV clone 13. (A) T-bet and PD-1 expression in D^{bgp276}-specific CD8⁺ T cells d60 p.i. Numbers denote frequency of gated population. (B) MFI of T-bet and PD-1 expression from cells in (A). (*p<0.05, **p<0.01; Paired t test)

These features of reduced exhaustion in Eomes-deficient CD8⁺ T cells were determined to be cell-intrinsic through the use of mixed bone-marrow chimeras (**Fig 3.5**). Thus, T-bet and Eomes support separate subpopulations of virus-specific CD8⁺ T cells during chronic viral infection.

3.3.4 T-bet and Eomes regulate distinct proliferative properties.

Previous studies in mice and humans suggest that proliferating and non-proliferating subpopulations of exhausted CD8⁺ T cells might exist during chronic infections (Hellerstein et al., 2003; Shin et al., 2007), but the regulation of this process is poorly understood. We hypothesized that T-bet^{hi} and Eomes^{hi} CD8⁺ T cells might have different *in vivo* proliferative dynamics. While T-bet^{hi} CD8⁺ T cells had low Ki-67 expression and BrdU incorporation, Eomes^{hi} CD8⁺ T cells expressed Ki-67 and incorporated BrdU at high frequencies in multiple anatomical sites, including the liver (**Fig 3.6A-C**). Using CFSE labeling to trace division history and associated changes in differentiation *in vivo*, we observed robust proliferation in Eomes^{hi} CD8⁺ T cells, while less CFSE dilution was found in the T-bet^{hi} population (**Fig 3.6D**). The modest proliferation of T-bet^{hi} cells was unlikely the result of limited access to antigen, since essentially all virus-specific memory CD8⁺ T cells divided extensively when transferred into chronically infected mice (**Fig 3.7**). In addition, T-bet and Eomes governed these distinct proliferative behaviors since deletion of T-bet increased CD8⁺ T cell Ki-67 expression and BrdU incorporation, while loss of Eomes diminished cell proliferation (**Fig 3.8, 3.9**). Thus, T-bet and Eomes regulate distinct CD8⁺ T cell pools with differential division during chronic infection.

Figure 3.6

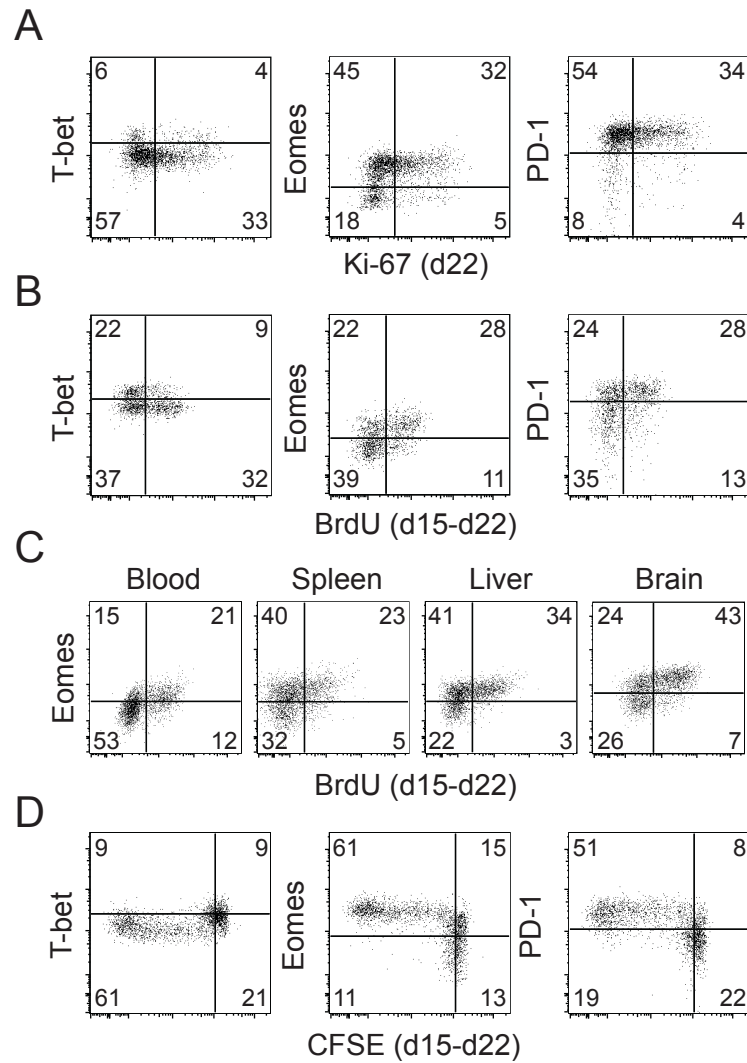


Figure 3.6. T-bet and Eomes subsets exhibit differential proliferation. (A) Ki-67 expression is increased in T-bet^{lo} Eomes^{hi} virus-specific CD8⁺ T cells. Flow cytometry of T-bet, Eomes, and PD-1 expression versus Ki-67 expression in gp33-specific CD8⁺ T cells 22 days post-infection. (B) BrdU incorporation is increased in T-bet^{lo} Eomes^{hi} virus-specific CD8⁺ T cells. Infected WT mice were injected with 2mg of BrdU daily from day 15 to day 21 post-infection. Flow cytometry of T-bet, Eomes, and PD-1 expression versus BrdU incorporation in gp33-specific CD8⁺ T cells 22 days post-infection. (C) BrdU incorporation occurs predominantly in the T-bet^{lo} Eomes^{hi} subset of virus-specific CD8⁺ T cells in multiple tissues. Infected mice were treated with BrdU as in (B). Plots display Eomes expression versus BrdU incorporation in gp276-specific CD8⁺ T cells from indicated organs. (D) Extensive proliferation is associated with Eomes expression. CD8⁺ T cells from CD45.2 infected mice were isolated 15 days post-infection, CFSE-labeled, and transferred into infection-matched CD45.1 hosts. One week post-transfer, virus-specific CD8⁺ T cells were analyzed for CFSE dilution. Plots show T-bet, Eomes, and PD-1 expression versus CFSE dilution in gp276-specific donor CD8⁺ T cells. Numbers indicate frequencies of gated populations.

Figure 3.7

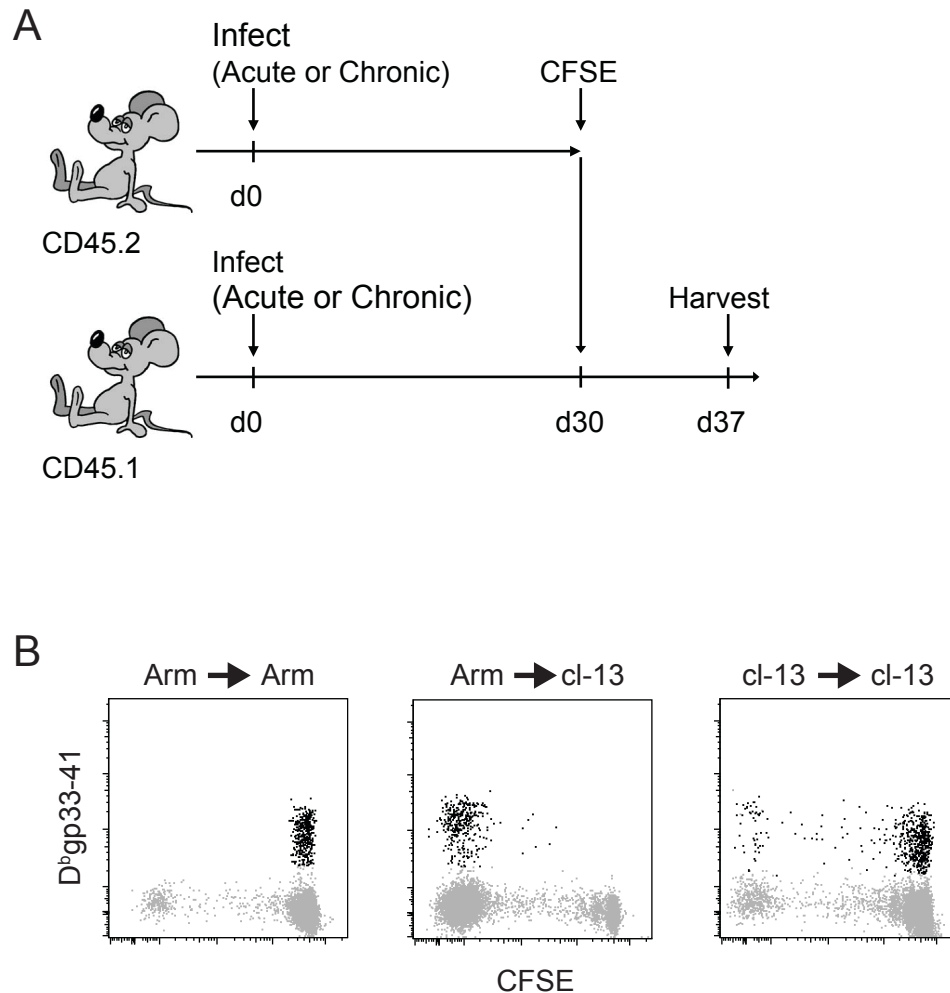


Figure 3.7. Lack of division in exhausted CD8⁺ T cells is not due to lack of proliferative (antigenic) stimuli. (A) CD8⁺ T cells from CD45.2 LCMV Armstrong-immune (d30⁺) or clone 13 chronically infected (d30) mice were isolated, CFSE-labeled, and transferred into LCMV Armstrong-immune (d30⁺) or chronically infected (d30) CD45.1 recipient mice. One week post-transfer, virus-specific CD8⁺ T cells were analyzed for CFSE dilution. (B) Plots show CFSE dilution in D^bgp33-specific donor CD8⁺ T cells.

Figure 3.8

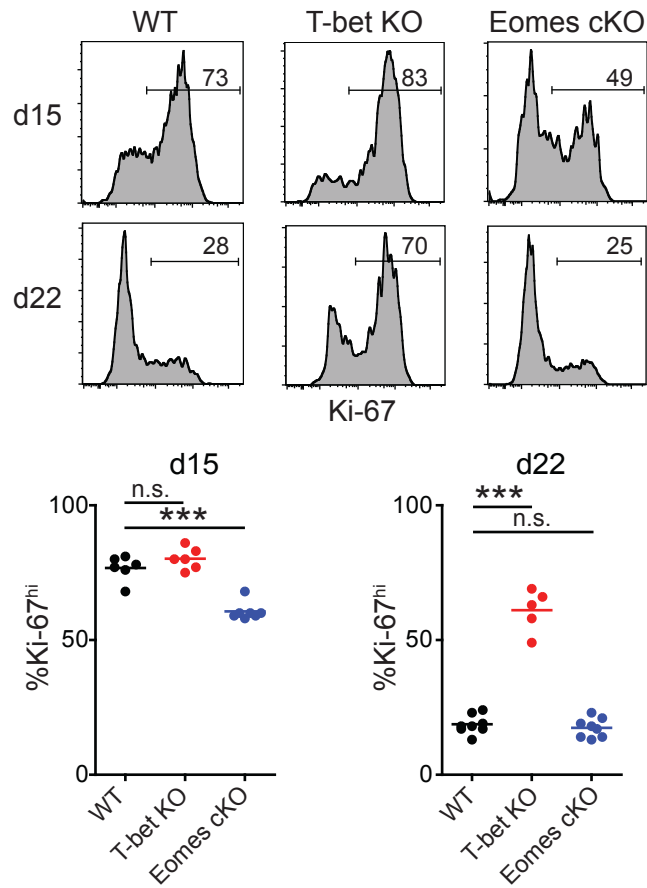


Figure 3.8. Deletion of either T-bet or Eomes leads to opposite effects on proliferation. Ki-67 expression in gp33-specific CD8⁺ T cells from WT, T-bet KO, and Eomes cKO mice at indicated days post-infection.

Figure 3.9

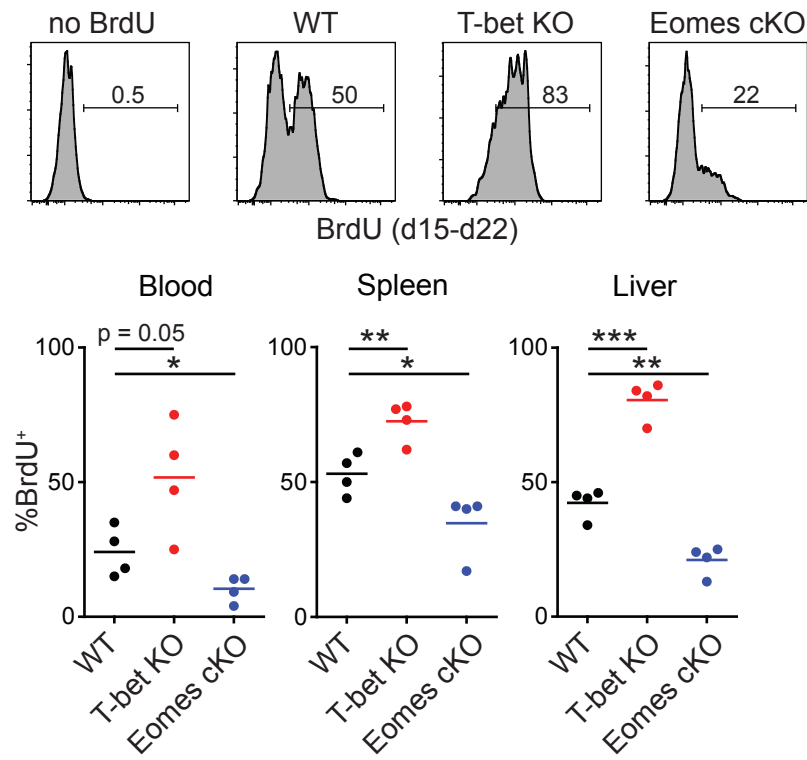


Figure 3.9. T-bet deficient CD8⁺ T cells exhibit enhanced proliferation, whereas Eomes deficient CD8⁺ T cells display reduced proliferation. BrdU incorporation in gp276-specific CD8⁺ T cells from WT, T-bet KO, and Eomes cKO mice. Mice were treated with BrdU and analyzed as in Fig 3.6.

3.3.5 T-bet^{hi} progenitors give rise to Eomes^{hi} progeny.

To trace lineage relationships between these subpopulations of exhausted CD8⁺ T cells, we first used expression of PD-1 as a surrogate surface marker of T-bet^{hi} and Eomes^{hi} subsets together with CFSE labeling to monitor proliferation and differentiation *in vivo* (**Fig 3.10A,B**). Following adoptive transfer to infection-matched mice, PD-1^{hi} (T-bet^{lo} Eomes^{hi}) CD8⁺ T cells divided modestly and retained high expression of PD-1 (**Fig 3.10C**). In contrast, PD-1^{int} (T-bet^{hi} Eomes^{lo}) CD8⁺ T cells demonstrated enhanced *in vivo* proliferation and this extensive division was associated with conversion to PD-1^{hi} (**Fig 3.10C**). Similar results were obtained using the Eomes^{gfp/+} reporter mouse (**Fig 3.11A**). Two weeks post-transfer, GFP⁺ CD8⁺ T cells maintained GFP expression while some GFP⁻ transitioned to GFP⁺ (**Fig 3.11B**). Thus, virus-specific CD8⁺ T cells appear to convert from T-bet^{hi} to Eomes^{hi} in a process coupled to extensive cell division. Once generated, however, the PD-1^{hi}Eomes^{hi}T-bet^{lo} subset had a reduced capacity to undergo additional proliferation *in vivo*. To test whether Eomes was essential during the T-bet^{hi} to Eomes^{hi} transition, virus-specific CD8⁺ T cells from chronically infected Eomes^{+/+} and Eomes^{ff} mice were treated *in vitro* with Tat-Cre and adoptively transferred into infection-matched mice (**Fig 3.12A**). The temporal loss of Eomes led to a reduction in the extensively divided CD8⁺ T cell population (**Fig 3.12B**), suggesting that Eomes plays a critical role in initiating or sustaining this proliferative event.

Figure 3.10

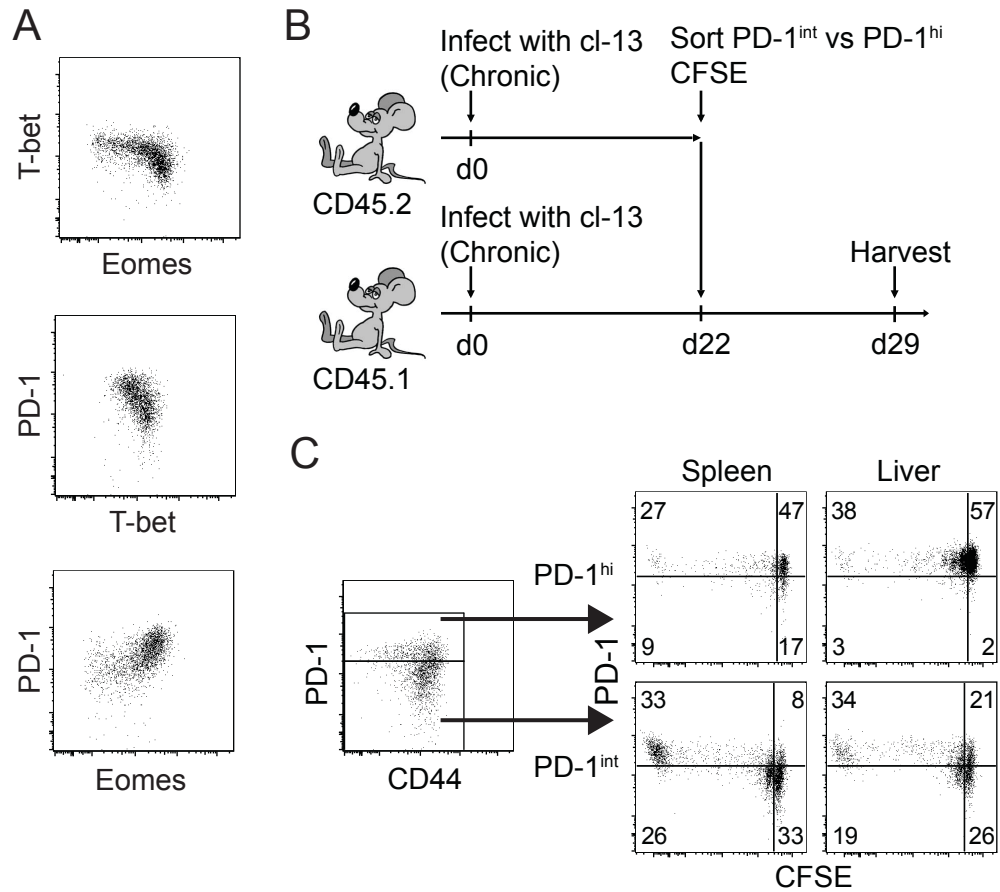


Figure 3.10. T-bet^{hi} Eomes^{lo} CD8⁺ T cells convert to T-bet^{lo} Eomes^{hi} CD8⁺ T cells. PD-1^{int} CD8⁺ T cells convert to PD-1^{hi} CD8⁺ T cells during extensive proliferation. (A) Relative expression of T-bet, Eomes, and PD-1 in D^bgp33-specific CD8⁺ T cells d21 post clone 13 infection. (B) CD8⁺ T cells from chronically infected CD45.2 mice were isolated d22 p.i., sorted based on PD-1 expression, CFSE-labeled, and transferred into infection-matched CD45.1 recipient mice. PD-1 expression and CFSE dilution were assessed 7 days post-transfer. (C) Plots display PD-1 expression versus CFSE dilution of donor gp276-specific CD8⁺ T cells. Numbers indicate frequencies of gated populations.

Figure 3.11

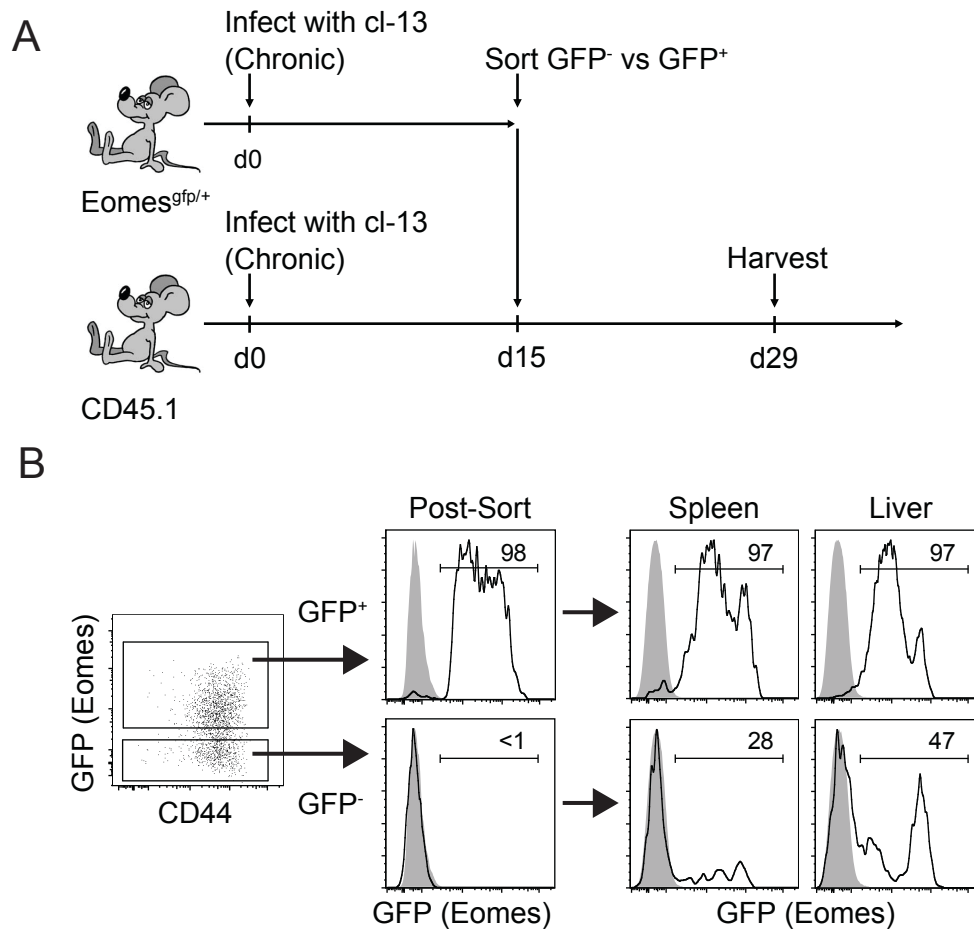


Figure 3.11. T-bet^{hi} Eomes^{lo} CD8⁺ T cells convert to T-bet^{lo} Eomes^{hi} CD8⁺ T cells. GFP⁻ CD8⁺ T cells from Eomes^{gfp/+} mice convert to GFP⁺ CD8⁺ T cells. (A) CD8⁺ T cells from chronically infected Eomes^{gfp/+} mice were isolated d15 p.i., sorted based on GFP expression, and transferred into infection-matched CD45.1 recipient mice. (B) Plots display GFP expression (open) in donor gp276-specific CD8⁺ T cells 2 weeks post-transfer. Numbers indicate frequencies of gated populations.

Figure 3.12

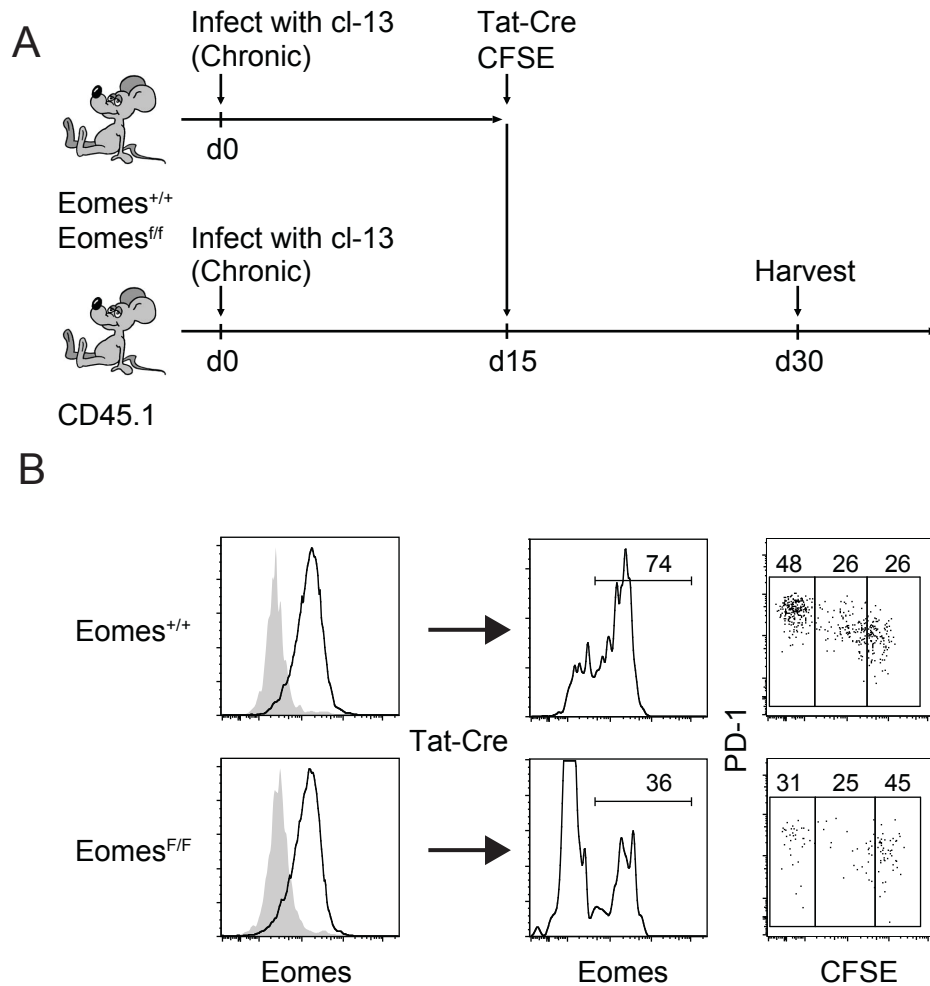


Figure 3.12. Eomes regulates the initiation or continuation of extensive CD8⁺ T cell proliferation. (A) CD8⁺ T cells from chronically infected Eomes^{+/+} or Eomes^{f/f} mice were isolated d15 p.i., treated with Tat-Cre *in vitro*, CFSE-labeled, and transferred into infection-matched CD45.1 recipient mice. Two weeks post-transfer, virus-specific CD8⁺ T cells were analyzed for CFSE dilution. (B) Plots display Eomes and PD-1 expression and CFSE dilution in donor gp276-specific CD8⁺ T cells. Numbers indicate frequencies of gated populations.

3.3.6 Loss of T-bet or Eomes leads to loss of the CD8⁺ T cell response and impaired viral control.

The absence of T-bet impedes the control of chronic viral infection, presumably due to a shift towards more exhausted CD8⁺ T cells (Kao et al., 2011). In light of the reduced features of exhaustion when Eomes was deleted (**Fig 3.4, 3.5**), we tested whether eliminating Eomes would improve control of chronic viral infection. In the absence of T-bet or Eomes, however, virus-specific CD8⁺ T cells could neither maintain the antiviral response (**Fig 3.13A, B**) nor limit viral replication (**Fig 3.13C, D**). Since each subpopulation failed to independently sustain an effective antiviral response, it was possible that the T-bet- and Eomes-dependent subsets might contribute distinct functions and cooperate to achieve viral control. To test this possibility, mixed chimeras containing both Eomes cKO and T-bet KO bone marrow together were examined. Combination of Eomes cKO (T-bet^{hi}) and T-bet KO (Eomes^{hi}) exhausted CD8⁺ T cells, however, did not improve viral control over either subset alone (**Fig 3.13E**). These data suggest that the major factor sustaining virus-specific CD8⁺ T cell responses and limiting viral replication during chronic infection is not independent functional attributes of T-bet^{hi} and Eomes^{hi} CD8⁺ T cells, but rather the lineage relationship between these subpopulations.

Figure 3.13

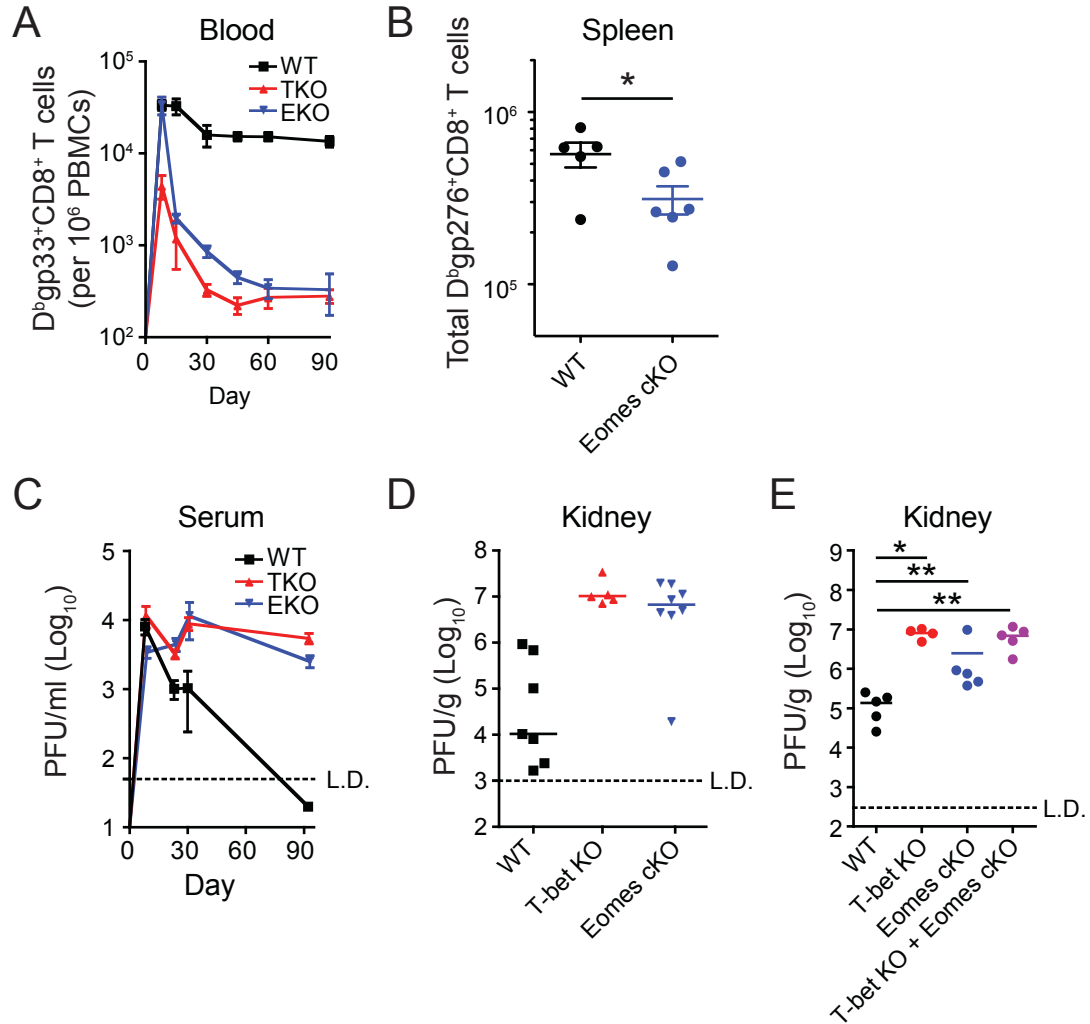


Figure 3.13. Deletion of T-bet or Eomes leads to impaired maintenance of the CD8⁺ T cell response and loss of viral control. (A) Longitudinal frequency of gp33-specific CD8⁺ T cells in the blood of infected mice. (B) Total gp276-specific CD8⁺ T cells in the spleens of infected mice at d30 p.i. (*p<0.05; Unpaired t test) (C) Longitudinal viral load in the serum of infected mice. (D) Viral load in the kidney at d90 p.i. (E) Viral load in kidneys of bone marrow (BM) chimeras transplanted with WT, TKO, EKO, or TKO and EKO BM at d90 p.i. (*p<0.05, **p<0.01; Mann Whitney)

3.3.7 Antigen drives the differentiation from T-bet^{hi} precursors to Eomes^{hi} progeny.

Persisting antigen appears to be essential for converting T-bet^{hi} precursors into the more terminal Eomes^{hi} progeny. Compared to cells adoptively transferred into a WT infection, transfer into mice chronically infected a variant of LCMV containing an epitope-destroying mutation at position 35 (V35A) in the gp33-41 epitope (Shin et al., 2007) selected for D^bgp33-specific CD8⁺ T cells with elevated T-bet but reduced Eomes and PD-1 expression (**Fig 3.14A**). If persisting antigen induces T-bet^{hi} precursors to continually give rise to highly proliferated Eomes^{hi} progeny, one might predict that prolonged, uncontrolled viral replication could lead to an eventual depletion of the T-bet^{hi}Eomes^{lo} precursor population. To test this possibility, CD4⁺ T cells were transiently depleted during LCMV clone 13 infection, a treatment that leads to lifelong high viral load (Matloubian et al., 1994). Sustained high viral loads caused an erosion of the *in vivo* proliferation of the Eomes^{hi} subset and loss of T-bet^{hi} precursors over time (**Fig 3.14B-D**), suggesting a progressive imbalance of T-bet^{hi} to Eomes^{hi} lineage repopulation.

3.3.8 Antigen drives the differentiation of T-bet^{hi} precursors to Eomes^{hi} progeny during hepatitis C infection.

To examine whether years of chronic viral infection strain CD8⁺ T cell renewal by continuous depletion of the T-bet^{hi} Eomes^{lo} precursor pool, we examined human subjects infected with hepatitis C virus (HCV). While 20-30% of HCV infections spontaneously resolve, most untreated patients experience a high viral burden for many years (Micallef et al., 2006). Over time, patients with uncontrolled viral replication have a gradual loss of systemic CD8⁺ T cell responses with an associated accumulation of highly exhausted T cells in the liver (Kasprowicz et al., 2008; Lechner et al., 2000; McMahan et al., 2010; Nakamoto et al., 2008), consistent with a dysregulated balance

between self-renewal and terminal differentiation. We therefore examined whether T-bet^{hi} Eomes^{lo} precursors could be found in patients with resolved versus chronic HCV infection and whether T-bet^{lo} Eomes^{hi} progeny accumulated in patients with active viral replication (**Fig 3.15A**). While HCV-specific CD8⁺ T cells in the blood displayed a trend toward higher Eomes expression in chronically infected subjects, there was equivalent T-bet expression in systemic responses from resolved or chronic infection (**Fig 3.15B**). In contrast, at the site of viral replication, there was substantial accrual of Eomes^{hi} HCV-specific CD8⁺ T cells and a relative depletion of the T-bet^{hi} subset during chronic infection compared to resolved infection (**Fig 3.15B**). Consistent with the observations in mice, chronic HCV infection is associated with few T-bet^{hi} precursors and a concurrent accumulation of Eomes^{hi} terminal progeny.

Figure 3.14

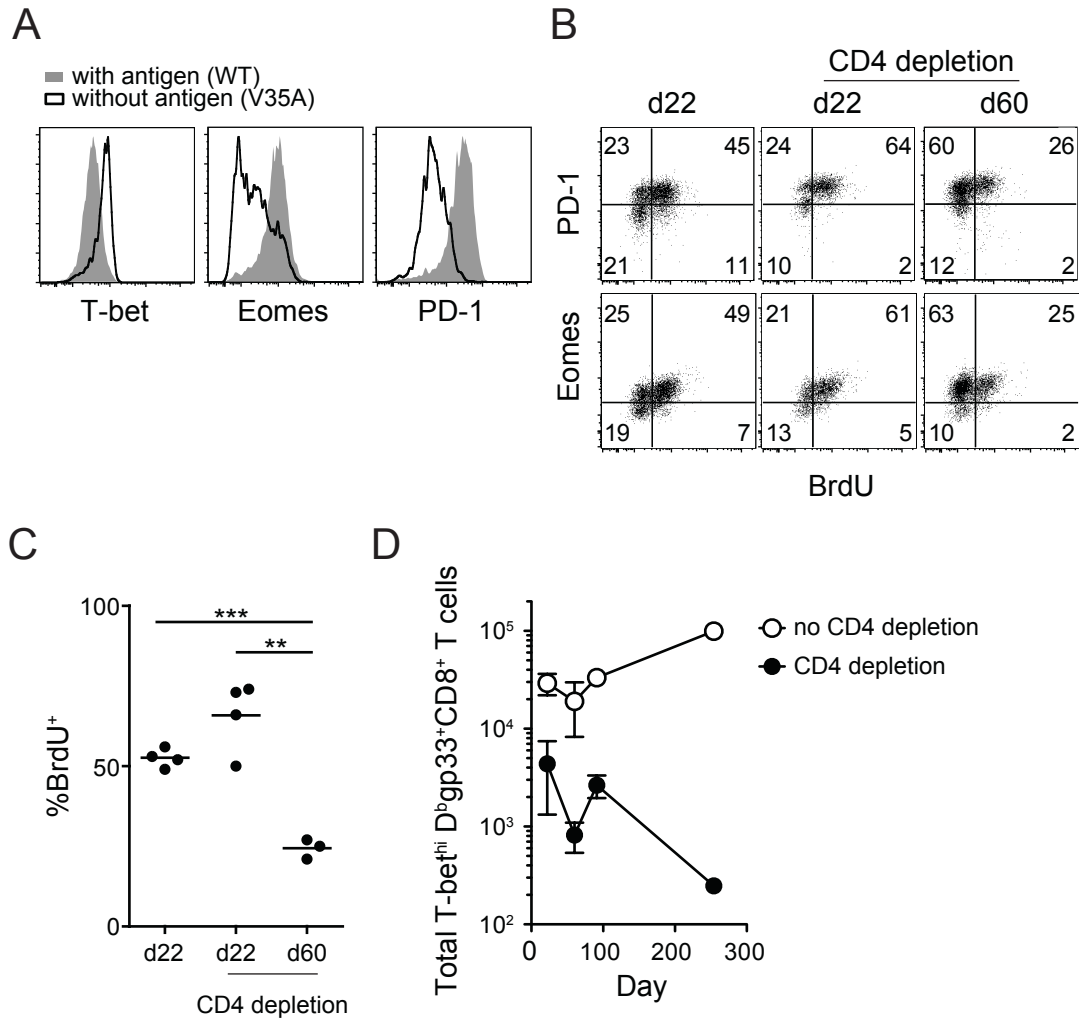


Figure 3.14. Persistent antigen may progressively deplete the T-bet^{hi} Eomes^{lo} CD8⁺ T precursor pool. (A) CD8⁺ T cells from infected mice were isolated at d8 p.i. and transferred to mice infected with either WT (shaded) or V35A (open) clone 13. Plots display T-bet, Eomes, and PD-1 expression in D^bgp33-specific CD8⁺ T cells in the presence (shaded) or absence (open) of antigen. (B) Eomes and PD-1 expression versus BrdU incorporation in D^bgp276-specific CD8⁺ T cells from WT mice infected with clone 13 with or without CD4 depletion. (C) Quantification of BrdU incorporation in (B). (D) Total T-bet^{hi} gp33-specific CD8⁺ T cells were assessed at indicated time-points in the spleens of chronically infected WT mice with or without CD4⁺ T cell depletion.

Figure 3.15

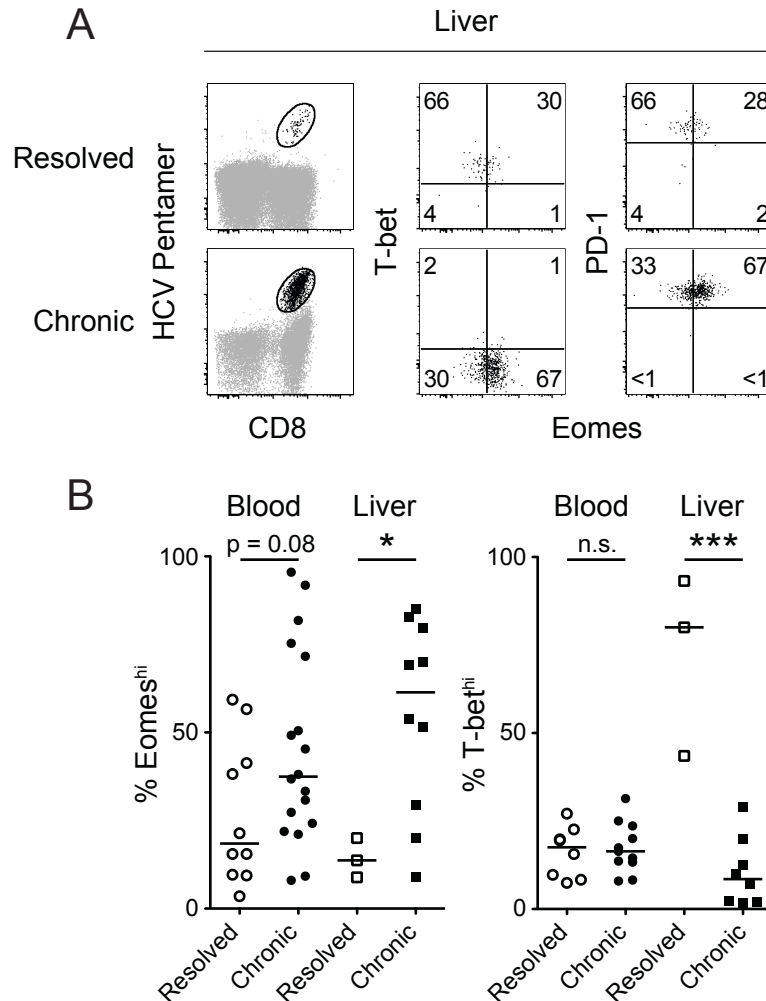


Figure 3.15. Antigen drives the differentiation of T-bet^{hi} precursors to Eomes^{hi} progeny during hepatitis C infection. (A) T-bet, Eomes, and PD-1 expression in intrahepatic HCV-specific CD8⁺ T cells for resolved and chronic infections. (B) Frequency of Eomes^{hi} and T-bet^{hi} HCV-specific CD8⁺ T cells in the blood and liver of resolved and chronic infections.

3.4 Discussion:

Memory CD8⁺ T cells generated after acutely resolved infection or successful vaccination are quiescent, but capable of self-renewal and production of differentiated effector progeny upon re-infection (Ciocca et al., 2012; Wherry et al., 2003b). In contrast, exhausted CD8⁺ T cells during chronic infections are continually activated by persisting antigen and undergo prolonged, extensive division (Casazza et al., 2001; Shin et al., 2007; Wherry et al., 2004). How the regenerative capacity of these cells develops and why it ultimately fails in pathological chronic infections has been unclear. Collectively, these studies suggest that, in the face of persisting antigen, virus-specific CD8⁺ T cells employ two homologous T-box transcription factors to maintain long-lasting antiviral immunity. This maintenance parallels progenitor-progeny dynamics of cells in other tissues organized according to proliferative hierarchies. In virus-specific CD8⁺ T cells, T-bet preserves the less exhausted T-bet^{hi} Eomes^{lo} precursor population that undergoes low rates of proliferation. In contrast, Eomes supports the more terminally exhausted T-bet^{lo} Eomes^{hi} population that has experienced extensive division, but has lost further proliferative potential. While unable to fully eradicate the virus, these two cell subsets act together to maintain a durable and partially effective CD8⁺ T cell response during chronic infection.

Other extracellular signals have also been reported to be essential for maintenance of antiviral CD8⁺ T cell responses during chronic infection. For example, IL-21 helps to preserve CD8⁺ T cell responses during chronic infection, presumably as one component of CD4⁺ T cell help (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009). Loss of IL-21 receptor signaling leads to a gradual loss of antiviral CD8⁺ T cell

responses (Elsaesser et al., 2009; Frohlich et al., 2009; Yi et al., 2009), however whether this is a uniform loss of the total population or a preferential loss of either the T-bet^{hi} or Eomes^{hi} sub-populations is not known. It is possible that IL-21 is an essential survival cytokine to sustain either the T-bet^{hi} or Eomes^{hi} pools. Alternatively, IL-21 could provide an important signal to enhance the proliferation of the transit amplifying cell that will repopulate the Eomes^{hi} pool. Although this question has not been fully explored, reduced BrdU incorporation and PD-1 expression early post infection in IL-21R KO cells from WT and IL-21R KO mixed bone marrow chimeras suggests that the Eomes^{hi} population may be preferentially lost without IL-21 signaling (Frohlich et al., 2009). This suggests that IL-21 may provide proliferative signals to the transit amplifying cell or survival signals to the terminal Eomes^{hi} population. This avenue of investigation could prove fruitful in understanding how extracellular cues help maintain antiviral CD8⁺ T cell responses, in particular if IL-21 is an important mechanism of CD4⁺ T cell help.

Despite functional impairment, exhausted CD8⁺ T cells are not inert. Rather, these cells continually constrain viral replication and/or drive viral epitope escape (Wherry, 2011). Design of interventions that would enhance CD8⁺ T cell responses to chronic viral infections or malignancy is limited by an incomplete understanding of CD8⁺ T cell population dynamics. Such treatments could augment the virus-specific population by either (1) expanding the T-bet^{hi} precursor population, (2) improving the expansion properties of the transient amplifying population converting from a T-bet^{hi} to an Eomes^{hi} state, or (3) protecting the terminally exhausted Eomes^{hi} population from cell death. Several therapies, however, have already been observed to expand the anti-viral CD8⁺ T cell population (Barber et al., 2006; Brooks et al., 2006; Ejrnaes et al., 2006; Pellegrini et

al., 2011). The most prominent of these is blockade of inhibitory receptor pathways, in particular the PD-1 pathway (Wherry, 2011).

While one might predict blockade of the PD-1 pathway would increase CD8⁺ T cell numbers by rescuing terminally exhausted CD8⁺ T cells, recent evidence instead suggests that PD-1 blockade preferentially affects either the T-bet^{hi} precursor population or a cell arising thereof. Examination of the effects of PD-1 blockade on exhausted CD8⁺ T cells separated by PD-1 expression demonstrated that PD-1 blockade has little to no impact on the properties of pre-formed PD-1^{hi} (Eomes^{hi}) progeny, but dramatically improves the ability of PD-1^{int} (T-bet^{hi}) precursors to expand and exert anti-pathogen activities (Blackburn et al., 2008). The benefits of PD-1 blockade could act directly on T-bet^{hi} cells and expand the precursor population, leading to a greater generation of Eomes^{hi} progeny. Alternatively, PD-1 blockade may act to augment the amplification phase as T-bet^{hi} progenitors expand and differentiate into Eomes^{hi} progeny. Determining which of these two processes may be the dominant effect of PD-1 blockade would suggest whether a short course of treatment would lead to a lasting effect on the CD8⁺ T cell response by permanently augmenting the progenitor population or only transiently boost the generation of antiviral progeny during the treatment period.

Several investigators have also begun to observe opportunities in manipulating multiple inhibitory and costimulatory pathways simultaneously (Blackburn et al., 2009; Butler et al., 2011; Jin et al., 2010; Matsuzaki et al., 2010; McMahan et al., 2010; Nakamoto et al., 2009; Raziorrouh et al., 2010; Sakuishi et al., 2010; Vezys et al., 2011; Woo et al., 2011; Zhou et al., 2011). However, not all interventions lead to a lasting improvement of the CD8⁺ T cell response. For example, coupling of PD-1 blockade to strong 41BB co-stimulation led to a dramatic, yet transient, augmentation of the CD8⁺ T

cell response (Vezys et al., 2011). The large CD8⁺ T cell contraction after therapy could be due to a temporary loss of the transient amplifying population. Alternatively, the strong 41BB stimulus may have led to depletion of the T-bet^{hi} precursor population. This depletion would likely have detrimental consequences with regard to long-term viral control. Thus, while manipulation of inhibitory and costimulatory pathways may effectively expand the anti-viral CD8⁺ T cell population, care may be required to avoid unbalancing this dynamic process.

A second question is how the T-bet^{hi} precursor population remains dormant despite the abundance of antigen present during a chronic viral infection. One possibility is that T-bet^{hi} precursors occupy specialized antigen-poor sites, preventing antigen-driven activation, proliferation, and differentiation. Alternatively T-bet^{hi} precursors may cell-intrinsically reduce T cell receptor signaling, entering a state of antigen ignorance. In either case, lack of antigen-driven activation may be an essential component to maintaining the T-bet^{hi} precursor population.

Consistent with this hypothesis and in contrast to exhausted CD8⁺ T cells, memory CD8⁺ T cells extensively proliferate when transferred into a chronic viral infection and do not appear to preserve an undivided precursor population. Furthermore, recent work has demonstrated that a small population of memory CD8⁺ T cells is unable to sustain a prolonged anti-viral response (West et al., 2011). Thus, if present in sufficient quantities memory CD8⁺ T cells are effectively programmed to protect the host in the acute phase of challenge; however, if the viral challenge cannot be contained and establishes a chronic infection, memory CD8⁺ T cells may not contain the optimal transcriptional programming to adapt to high levels of persistent antigen.

The inability of memory CD8⁺ T cells to sustain an anti-viral response to a chronic infection may have implications for vaccine development. Vaccines have historically achieved efficacy through preventing productive infections. However, chronic viral infections such as HIV and HCV have eluded such vaccine strategies. Moreover, as noted above, failure to limit viral replication may lead to loss of a vaccine-induced CD8⁺ T cell population, rendering the host in a worse state than unvaccinated individuals. Recent investigations, however, may have provided an alternative vaccine strategy. Using a CMV-based vector for SIV vaccination, Hansen and colleagues elicited immune responses that did not prevent SIV infection, but instead quickly limited viral replication (Hansen et al., 2011). While SIV was not immediately eliminated, brief spikes in serum viral load occurred with reduced frequency over time, suggesting a prolonged and active CD8⁺ T cell response that progressively controlled viral replication. Many differences exist between the CMV-based platform and more common prime-boost regimes. However, delivery of persistent antigen by the CMV-based vector may induce the formation of a T-bet^{hi} CD8⁺ T cell precursor pool that resists antigen-induced activation and proliferation, allowing CD8⁺ T cells to generate a sustained and effective anti-viral response. Thus, in addition to strategies that may elicit sterilizing immunity, vaccine platforms that employ persistent antigen may offer a second approach to combat chronic viral infections.

CHAPTER 4: Concluding remarks and future directions

4.1 Overview of Results.

Eomes has been reported to direct the differentiation of NK (Gordon et al., 2012) and CD8⁺ T cells (Banerjee et al., 2010; Intlekofer et al., 2008; Intlekofer et al., 2005). What have remained less clear, however, are the specific features of the CD8⁺ T cell response that correlate with Eomes expression. In **Chapter 2** of this thesis, Eomes expression was not associated with a differential cytotoxic potential of *in vivo* generated effector cells. Nevertheless, Eomes expression was associated with enhanced basal homeostatic proliferation and Eomes protein was essential to maintain this homeostatic division, a finding previously suggested (Banerjee et al., 2010; Intlekofer et al., 2005) but which had yet to be formally demonstrated. Thus, the description and characterization of the Eomes^{gfp/+} mouse has provided a solid foundation for exploration into the biology of Eomesodermin in both **Chapters 2 & 3**. This reagent may still provide opportunities to further discovery (discussed below).

In CD8⁺ T cell responses to acutely resolved infections, Eomes supports the long-lived, self-renewing memory population. In contrast, during the course of a chronic viral infection, Eomes was found in **Chapter 3** to support the generation and maintenance of a population of short-lived, terminally differentiated cells. Eomes helps to maintain this antiviral population by supporting the extensive cell division necessary to repeatedly repopulate this terminal pool from T-bet^{hi} precursors. Thus, depending on the duration of infection, T-box transcription factors appear to support either a long-lived progenitor pool or a terminally differentiated antiviral population.

The critical balance between progenitors and progeny in exhausted CD8⁺ T cells indicates a vital role for proper regulation of antiviral lymphocyte dynamics during chronic infections. The ability to define and monitor progenitors and progeny over time might provide an opportunity to predict the collapse of long-term control of chronic infections. Finally, delineating the molecular coordination of this process provides a framework for prophylactic or therapeutic strategies to improve the durability and regenerative capacity of antiviral T cells during persisting infections.

4.2 Distinct or identical activity of T-box factors in response to acutely resolved vs chronic viral infection.

Previous work has associated Eomes transcriptional activity with highly functional CD8⁺ T cell responses, either through the acquisition of effector functions (Intlekofer et al., 2008; Pearce et al., 2003) or through maintaining the long-lived and self-renewing central memory CD8⁺ T cell population after an acutely resolved infection (Banerjee et al., 2010; Intlekofer et al., 2005). Alternatively, T-bet activity is often associated with terminal differentiation (Intlekofer et al., 2007; Joshi et al., 2007). Thus, the findings in **Chapter 3** that Eomes is highly expressed in more terminally differentiated and dysfunctional CD8⁺ T cells during chronic infection, while T-bet supports a less-differentiated precursor population, may appear paradoxical. One possibility is that distinct cofactors arise in acute versus chronic infections and differentially modulate the transcriptional activity of T-box factors. Consistent with this hypothesis, Bcl6 appears to convert T-bet from a transcriptional activator to a repressor, at least for a select set of target genes (Oestreich et al., 2011). The pathways and functions that these T-box factors activate or repress may display a large degree of context-dependent variation.

An alternate or complimentary hypothesis, however, is that Eomes may regulate a common pathway that is shared across multiple cell types and environmental stimuli. During embryonic development, Eomes regulates both trophoblast formation (Russ et al., 2000) and neuronal development (Arnold et al., 2008). In the immune system, Eomes appears to regulate the maturation of NK cells (Gordon et al., 2012), self-renewing central memory CD8⁺ T cells (Banerjee et al., 2010), and aberrant double-negative T cells in the setting of autoimmune Autoimmune Lymphoproliferative Syndrome (ALPS) (Kinjyo et al., 2010). One consistent feature in these disparate settings is that Eomes is expressed in cells undergoing proliferation. Furthermore, deletion of Eomes leads to the loss of this proliferative population (Arnold et al., 2008; Banerjee et al., 2010; Gordon et al., 2012; Kinjyo et al., 2010; Russ et al., 2000). Thus, Eomes may activate specific pathways – DNA damage responses, metabolic changes, etc. – that are essential for maintaining cells during these periods of proliferation. Understanding how Eomes might regulate cell proliferation may lead to novel or improved therapies directed at eliminating unwanted populations such as in ALPS or bolstering desired populations such as in anti-viral or anti-tumor CD8⁺ T cell responses.

4.3 Use of a fluorescent reporter to identify transcriptional targets and regulators of Eomesodermin.

Identification of the transcriptional targets of Eomes during acute and chronic infection would begin to address whether these T-box factors have conserved activity with situational manifestations or context dependent activity. Two approaches could prove to be fruitful in addressing this question. First, chromatin immunoprecipitation of Eomes from antiviral CD8⁺ T cells at different stages of differentiation (effector, memory, and exhausted) could determine whether Eomes may regulate the same or distinct gene

sets in response to acutely resolved or chronic infections. While Eomes might bind to and thereby regulate distinct gene sets in acutely resolved and chronic viral infections, this differential binding could simply be a result of chromatin accessibility, not differential targeting. Consistent with this hypothesis, T-box transcription factors appear to have equal binding of consensus sequences regardless of context. For example, T-box factors from separate subfamilies can bind the same set of target genes (Lewis et al., 2007), suggesting that association with potentially distinct cofactors does not limit binding to transcriptional targets. Similarly, T-bet has been reported to bind the same set of target genes in a variety of lymphocytes regardless of transcriptional activity at such targets (Beima et al., 2006). These findings suggest that the context dependent cofactors for T-box factors (such as those that vary between lymphocyte lineages) may not direct binding to distinct transcriptional targets, but rather may modulate the transcriptional activity of T-box targets. Thus, while promising, chromatin immunoprecipitation may be only one or several technologies that need to be employed to determine how T-box factors have context dependent functions.

A complimentary approach to investigate the differential activity of Eomes in acutely resolved versus chronic infections would employ the Eomes^{gfp/+} reporter mouse characterized in **Chapter 2**. Whole genome expression profiling has been used to define the transcriptional signature at different stages of cellular differentiation (Ivanova et al., 2002; Novershtern et al., 2011; Phillips et al., 2000; Wherry et al., 2007). In a similar manner, gene expression analysis of GFP⁺ and GFP⁻ CD8⁺ T cells from Eomes^{gfp/fl} mice with or without a CD4-Cre could not only define the transcriptional program associated with Eomes expression but also begin to differentiate the transcriptional program

activated upstream or in parallel to Eomes expression from the set of genes that are regulated downstream of Eomes.

Specifically, the set of differentially expressed genes between GFP⁺ and GFP⁻ Eomes^{gfp/+} CD8⁺ T cells would comprise the transcriptional network associated with Eomes expression, however, this gene set is a combination of genes that are activated upstream or in parallel to Eomes transcription and genes that are directly regulated or downstream of Eomes transcriptional activity. Transcriptional profiling of GFP⁺ and GFP⁻ Eomes^{gfp/-} CD8⁺ T cells would assist in disaggregating these two gene sets. The differentially expressed genes between GFP⁺ and GFP⁻ Eomes^{gfp/-} CD8⁺ T cells would define the gene set activated upstream and in parallel to Eomes activation. In contrast, the differentially expressed gene set between GFP⁺ CD8⁺ T cells with or without Eomes protein would define the transcriptional program downstream of Eomes transcriptional activity. Disaggregating these two gene sets could illuminate new opportunities for therapeutic intervention in settings where Eomes activity may need to be enhanced, such as CD8⁺ T cell responses to viral infection (Banerjee et al., 2010; Intlekofer et al., 2008; Intlekofer et al., 2005), or reduced, such as double negative T cells in ALPS (Kinjyo et al., 2010).

4.4 Senescence versus Exhaustion: distinct or overlapping processes?

Using the LCMV model of chronic viral infection, T cell exhaustion was defined by impaired antiviral responses, including reduced proliferation and effector functions (Zajac et al., 1998). The development of exhaustion is dependent on the persistence of antigen (Mueller and Ahmed, 2009) and displays a gradient of dysfunction that correlates with the length or intensity of antigen exposure (Wherry et al., 2003a). In addition to prolonged antigen exposure, immunoregulation is a second fundamental

component that limits effective and functional responses. As mentioned in **Chapter 1**, specific immunoregulatory cytokines, such as IL-10 (Brooks et al., 2008; Brooks et al., 2006) or TGF-beta (Tinoco et al., 2009) have been reported to limit CD8⁺ T cell responses and effective viral clearance. Similarly, expression of inhibitory receptors are thought to impair optimal antiviral immunity, since enhanced expression of inhibitory receptors correlates with reduced functionality and blockade of these signaling pathways leads to improved T cell function. While the examination and potential reversal of CD8⁺ T cell exhaustion has largely focused on the loss of effector functions, the revival of proliferative capacity is a prominent feature of restored antiviral responses. Whether these two features are intertwined and causally linked is unclear.

Senescence is the loss of further proliferative potential due to either telomere shortening or the DNA damage response to irreparable DNA, which often develops after a period of extensive proliferation (Akbar and Henson, 2011). Replicative senescence of CD8⁺ T cells is associated with aging, leading to poor antiviral responses in aged mice or humans (Akbar and Henson, 2011; Decman et al., 2010). These responses are best characterized by a reduced expansion in response to antigenic stimuli (Akbar and Henson, 2011; Decman et al., 2010), however, they also contain other dysfunctional features, including increased inhibitory receptor expression (Decman et al., 2012). Thus, senescence of CD8⁺ T cells share a number a features with T cell exhaustion, prompting speculation whether the two processes overlap.

Consistent with this hypothesis, previous analyses of CD8⁺ T cells from HIV-infected subjects revealed an elevated proliferative history as measured by T cell Receptor Excision Circles (Brenchley et al., 2003), markers associated with shortened telomeres (Brenchley et al., 2003), and elevated incorporation of deuterated glucose into

DNA (Hellerstein et al., 1999; Hellerstein et al., 2003; Ladell et al., 2008; McCune et al., 2000; Mohri et al., 2001). In addition, HIV-specific CD8⁺ T cells have reduced proliferative potential *in vitro* after antigenic stimulation (Brenchley et al., 2003). Lastly, the transcriptional profiles of age-associated senescence and exhaustion share a similar transcriptional signature (Decman et al., 2012).

The data presented in **Chapter 3** contextualize the apparent overlap between replicative senescence and functional exhaustion. During chronic infection, Eomes expression defines a population of CD8⁺ T cells that display reduced effector function, increased inhibitory receptor expression, extensive replicative history, and reduced proliferative potential. This Eomes^{hi} population constitutes the majority of the antiviral response during periods of high viral load when dysfunctional responses are most apparent. Thus, previous analysis of virus-specific CD8⁺ T cell populations during chronic infection may have principally described the Eomes^{hi}, more terminally differentiated and senescent population, and likely overlooked the proliferative potential of the T-bet^{hi} precursor pool.

Whether inhibitory receptor expression increases and effector functions decrease as a direct correlate of division history in the Eomes^{hi} pool remains to be determined. Nevertheless, it is likely that extensive division history in both exhaustion and senescence correlates with reduced proliferative potential and effector functions. Whether this process is controlled by the same molecular mechanisms remains to be explored.

4.5 Divergent development of memory and exhaustion.

In **Chapter 3**, a long-lived T-bet^{hi} progenitor pool was determined to sustain the antiviral CD8⁺ T cell response. How this progenitor pool develops is unclear. What has been demonstrated, however, is the progressive divergence of memory and exhaustion. CD8⁺ T cells appear to have equal potential for memory or exhaustion early in the antiviral response (day 8 post-infection) depending on the resolution or persistence of infection. This apparent plasticity is restricted to the memory precursor population, as terminal effectors do not persist in the face of persistent antigen (Angelosanto et al., 2012); however, it remains to be determined whether (1) memory precursors are homogeneously receptive to instructive signals associated with resolution or persistence of viral infection or (2) subpopulations of memory precursors are pre-programmed for these two distinct outcomes and respond to selective signals that result from resolution or persistence of infection.

4.6 Similarities and differences between recall responses of memory and maintenance of exhaustion.

A major question arising from this work is whether the continual repopulation of the Eomes^{hi} pool from T-bet^{hi} precursors parallels recall responses by memory cells against re-exposure to cognate antigen. In the most fundamental framework, these two processes are quite similar. In both cases, in response to antigenic stimulation, a long-lived precursor population contains the capacity to differentiate, proliferate, and (re)generate a larger, short-lived population. In addition, a lengthy and repeated repopulation of the short-lived pool drains the renewal and proliferative capacity of the long-lived population and eventually impairs future proliferative events. Thus, conceptually, maintenance of exhausted CD8⁺ T cell responses by the T-bet^{hi} precursor

pool parallels the essential function of long-lived memory CD8⁺ T cells in providing lifelong immunity to acutely resolved infections.

Previous reports (West et al., 2011) in conjunction with the data presented in **Chapter 3**, however, suggest important differences between memory CD8⁺ T cells and the T-bet^{hi} precursor population during chronic infection. In particular, the relative responsiveness to antigen as measured by the extent of subsequent proliferation is markedly distinct between these two populations. In the presence of a high antigen burden during chronic infection, a small percentage of T-bet^{hi} precursors intermittently give rise to extensively divided Eomes^{hi} progeny. While a similar phenomenon appears to occur with memory CD8⁺ T cells after a small and relatively transient encounter with antigen (Ciocca et al., 2012), memory cells transferred into a chronic infection almost uniformly proliferate extensively and display little renewal behavior. Consistent with impaired renewal of memory CD8⁺ T cells in the context of prolonged high antigen exposure, memory CD8⁺ T cells appear incapable of sustaining an antiviral response during a chronic viral infection when viral control is not rapidly achieved (West et al., 2011). Thus, the T-bet^{hi} precursor population displays a resistance to antigen-induced activation, proliferation, and differentiation that may be essential to maintaining an antiviral response during chronic infection. The distinctions between these two types of long-lived, precursor populations may provide important avenues to prophylactically or therapeutically intervene in one process without altering the other.

What determines this relative “antigen ignorance” in T-bet^{hi} precursors during chronic infection is not well understood. One possibility is that these precursors occupy select niches in the immune compartment that do not efficiently or adequately present viral antigens, thus providing a cell-extrinsic method to preserve antigen ignorance. A

second, cell-intrinsic mechanism for antigen ignorance could be down modulation of TCR-signaling pathways. While this second mechanism may be more appealing, how this would occur is unclear. Although inhibitory receptors are likely candidates to temper TCR signaling in the T-bet^{hi} population, these receptors are more highly expressed on the Eomes^{hi} population than the T-bet^{hi} population. Thus, alternative mechanisms for attenuating TCR-signaling may be more likely to maintain the state of relative antigen ignorance of the T-bet^{hi} population. For example, the components of TCR-signaling pathways may be differentially expressed or differentially regulated between precursors and progeny. Alternatively, the distinct transcriptional status of these two populations may lead to distinct transcriptional responses to TCR signaling.

Differential responsiveness to antigen receptor signaling is not unique to exhausted CD8⁺ T cells. For example, strong antigen receptor signals of developing single positive thymocytes or immature B cells in the bone marrow lead to negative selection and apoptosis. In contrast, once those cells exit the thymus or bone marrow and mature into naïve T or B cells in the periphery, strong antigen receptor stimuli leads to robust proliferation and further differentiation. How the same signaling events lead to dramatically distinct cellular outcomes, death or proliferation, remains unresolved. Similar to exhausted CD8⁺ T cells one hypothesis is that the signaling pathway downstream of the proximal signaling events may be differentially structured, leading to the opposing outcomes. Alternatively, the transcriptional program operating during T and B cell development may differentially modulate the activity of the downstream transcription factors, such as NFAT, NF- κ B, and others. As a result, further exploration into the mechanisms behind the differential responses to TCR signaling in exhausted

CD8⁺ T cells may inform other scenarios of disparate reactions to antigen receptor signals.

4.7 Does asymmetric cell division contribute to CD8⁺ T cell responses during chronic viral infection?

In response to antigenic stimuli or other external cues, lymphocytes have been proposed to undergo asymmetric cell division (ACD) to generate two daughter cells with distinct properties and outcomes (Barnett et al., 2012; Chang et al., 2011; Chang et al., 2007; Ciocca et al., 2012; Thaunat et al., 2012). This process is of particular relevance to memory lymphocytes that are required to generate a large, short-lived population for pathogen clearance while renewing the memory pool for lifelong immunity (Ciocca et al., 2012). In a similar manner during chronic infection, T-bet^{hi} precursors delineated in **Chapter 3** are required to produce daughter cells that seed the Eomes^{hi} population while concurrently preserving the precursor pool. Whether exhausted CD8⁺ T cells employ ACD to preserve T-bet^{hi} precursors while repopulating Eomes^{hi} progeny is not known.

If ACD were one mechanism to sustain antiviral responses to chronic infections, one would predict that T-bet^{hi} precursors would have relatively high rates of ACD to simultaneously generate one cell the T-bet^{hi} pool while giving rise to a second transit amplifying cell that would undergo a series of subsequent symmetric divisions that would repopulate the Eomes^{hi} pool. If this were the case, specific fate determinants would be asymmetrically distributed during this process. In particular, specific transcription factors that regulate exhausted CD8⁺ T cells could be differentially inherited, such as T-bet or Eomes as well as Blimp1 (Shin et al., 2009), BATF (Quigley et al., 2010), STAT3 (Siegel et al., 2011), NFAT (Agnellini et al., 2007), or a number of other factors upregulated in exhausted CD8⁺ T cells (Wherry et al., 2007).

4.8 Summary

When the work described here was initiated, the transcriptional regulation and population dynamics of CD8⁺ T cell responses to chronic viral infections were not well understood. The identification of precursors and progeny in the CD8⁺ T cell response should provide a framework for future investigations into disease progression and immunologic manipulation during chronic infection. Even so, the work presented here may also emphasize how investigators should use the caution before defining a transcriptional program by only one or two transcription factors, as T-bet and Eomes, like many other transcription factors, appear to act in exquisitely context dependent manners.

BIBLIOGRAPHY

Centers for Disease Control and Prevention (2010). Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. *MMWR Morb Mortal Wkly Rep* 59, 1057-1062.

Centers for Disease Control and Prevention (2011). HIV Surveillance Report, 2009; vol. 21. <http://www.cdc.gov/hiv/topics/surveillance/resources/reports/>. Accessed July 1st, 2012.

Agnellini, P., Wolint, P., Rehr, M., Cahenzli, J., Karrer, U., and Oxenius, A. (2007). Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8+ T cell functions during chronic viral infection. *Proc Natl Acad Sci U S A* 104, 4565-4570.

Ahmed, R., Salmi, A., Butler, L.D., Chiller, J.M., and Oldstone, M.B. (1984). Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160, 521-540.

Akbar, A.N., and Henson, S.M. (2011). Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol* 11, 289-295.

Angelosanto, J.M., Blackburn, S.D., Crawford, A., and Wherry, E.J. (2012). Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. *J Virol*.

Araki, K., Turner, A.P., Shaffer, V.O., Gangappa, S., Keller, S.A., Bachmann, M.F., Larsen, C.P., and Ahmed, R. (2009). mTOR regulates memory CD8 T-cell differentiation. *Nature* 460, 108-112.

Arnold, S.J., Huang, G.J., Cheung, A.F., Era, T., Nishikawa, S., Bikoff, E.K., Molnar, Z., Robertson, E.J., and Groszer, M. (2008). The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone. *Genes Dev* 22, 2479-2484.

Arnold, S.J., Sugnaseelan, J., Groszer, M., Srinivas, S., and Robertson, E.J. (2009). Generation and analysis of a mouse line harboring GFP in the Eomes/Tbr2 locus. *Genesis* 47, 775-781.

Atreya, I., Schimanski, C.C., Becker, C., Wirtz, S., Dornhoff, H., Schnurer, E., Berger, M.R., Galle, P.R., Herr, W., and Neurath, M.F. (2007). The T-box transcription factor eomesodermin controls CD8 T cell activity and lymph node metastasis in human colorectal cancer. *Gut* 56, 1572-1578.

Banerjee, A., Gordon, S.M., Intlekofer, A.M., Paley, M.A., Mooney, E.C., Lindsten, T., Wherry, E.J., and Reiner, S.L. (2010). Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche. *J Immunol* 185, 4988-4992.

- Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., and Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682-687.
- Barnett, B.E., Ciocca, M.L., Goenka, R., Barnett, L.G., Wu, J., Laufer, T.M., Burkhardt, J.K., Cancro, M.P., and Reiner, S.L. (2012). Asymmetric B cell division in the germinal center reaction. *Science* **335**, 342-344.
- Basson, C.T., Bachinsky, D.R., Lin, R.C., Levi, T., Elkins, J.A., Soultz, J., Grayzel, D., Kroumpouzou, E., Traill, T.A., Leblanc-Straceski, J., *et al.* (1997). Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet* **15**, 30-35.
- Becker, T.C., Coley, S.M., Wherry, E.J., and Ahmed, R. (2005). Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J Immunol* **174**, 1269-1273.
- Becker, T.C., Wherry, E.J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A., and Ahmed, R. (2002). Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* **195**, 1541-1548.
- Beeman, E.A. (2007). Charles Armstrong M.D.: A biography (Office of History, National Institutes of Health).
- Beima, K.M., Miazgowiec, M.M., Lewis, M.D., Yan, P.S., Huang, T.H., and Weinmann, A.S. (2006). T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. *J Biol Chem* **281**, 11992-12000.
- Blackburn, S.D., Crawford, A., Shin, H., Polley, A., Freeman, G.J., and Wherry, E.J. (2010). Tissue-specific differences in PD-1 and PD-L1 expression during chronic viral infection: implications for CD8 T-cell exhaustion. *J Virol* **84**, 2078-2089.
- Blackburn, S.D., Shin, H., Freeman, G.J., and Wherry, E.J. (2008). Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc Natl Acad Sci U S A* **105**, 15016-15021.
- Blackburn, S.D., Shin, H., Haining, W.N., Zou, T., Workman, C.J., Polley, A., Betts, M.R., Freeman, G.J., Vignali, D.A., and Wherry, E.J. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* **10**, 29-37.
- Blattman, J.N., Wherry, E.J., Ha, S.J., van der Most, R.G., and Ahmed, R. (2009). Impact of epitope escape on PD-1 expression and CD8 T-cell exhaustion during chronic infection. *J Virol* **83**, 4386-4394.
- Bongers, E.M., Duijf, P.H., van Beersum, S.E., Schoots, J., Van Kampen, A., Burckhardt, A., Hamel, B.C., Losan, F., Hoefsloot, L.H., Yntema, H.G., *et al.* (2004).

Mutations in the human TBX4 gene cause small patella syndrome. *Am J Hum Genet* 74, 1239-1248.

Borrow, P., and Oldstone, M.B. (1992). Characterization of lymphocytic choriomeningitis virus-binding protein(s): a candidate cellular receptor for the virus. *J Virol* 66, 7270-7281.

Brahmer, J.R., Tykodi, S.S., Chow, L.Q., Hwu, W.J., Topalian, S.L., Hwu, P., Drake, C.G., Camacho, L.H., Kauh, J., Odunsi, K., *et al.* (2012). Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. *N Engl J Med*.

Brenchley, J.M., Karandikar, N.J., Betts, M.R., Ambrozak, D.R., Hill, B.J., Crotty, L.E., Casazza, J.P., Kuruppu, J., Migueles, S.A., Connors, M., *et al.* (2003). Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8⁺ T cells. *Blood* 101, 2711-2720.

Brooks, D.G., Ha, S.J., Elsaesser, H., Sharpe, A.H., Freeman, G.J., and Oldstone, M.B. (2008). IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection. *Proc Natl Acad Sci U S A* 105, 20428-20433.

Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., and Oldstone, M.B. (2006). Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12, 1301-1309.

Buchbinder, S.P., Mehrotra, D.V., Duerr, A., Fitzgerald, D.W., Mogg, R., Li, D., Gilbert, P.B., Lama, J.R., Marmor, M., Del Rio, C., *et al.* (2008). Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 372, 1881-1893.

Butler, N.S., Moebius, J., Pewe, L.L., Traore, B., Doumbo, O.K., Tygrett, L.T., Waldschmidt, T.J., Crompton, P.D., and Harty, J.T. (2011). Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol* 13, 188-195.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific CD8⁺ T cells during an acute virus infection. *Immunity* 8, 167-175.

Cao, W., Henry, M.D., Borrow, P., Yamada, H., Elder, J.H., Ravkov, E.V., Nichol, S.T., Compans, R.W., Campbell, K.P., and Oldstone, M.B. (1998). Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science* 282, 2079-2081.

Casazza, J.P., Betts, M.R., Picker, L.J., and Koup, R.A. (2001). Decay kinetics of human immunodeficiency virus-specific CD8⁺ T cells in peripheral blood after initiation of highly active antiretroviral therapy. *J Virol* 75, 6508-6516.

Chang, J.T., Ciocca, M.L., Kinjyo, I., Palanivel, V.R., McClurkin, C.E., Dejong, C.S., Mooney, E.C., Kim, J.S., Steinell, N.C., Oliaro, J., *et al.* (2011). Asymmetric proteasome segregation as a mechanism for unequal partitioning of the transcription factor T-bet during T lymphocyte division. *Immunity* 34, 492-504.

- Chang, J.T., Palanivel, V.R., Kinjyo, I., Schambach, F., Intlekofer, A.M., Banerjee, A., Longworth, S.A., Vinup, K.E., Mrass, P., Oliaro, J., *et al.* (2007). Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* **315**, 1687-1691.
- Chang, L.Y., Chang, I.S., Chen, W.J., Huang, Y.C., Chen, G.W., Shih, S.R., Juang, J.L., Shih, H.M., Hsiung, C.A., Lin, T.Y., and Huang, L.M. (2008). HLA-A33 is associated with susceptibility to enterovirus 71 infection. *Pediatrics* **122**, 1271-1276.
- Chapman, S.J., and Hill, A.V. (2012). Human genetic susceptibility to infectious disease. *Nat Rev Genet* **13**, 175-188.
- Chen, T., Heller, E., Beronja, S., Oshimori, N., Stokes, N., and Fuchs, E. (2012). An RNA interference screen uncovers a new molecule in stem cell self-renewal and long-term regeneration. *Nature* **485**, 104-108.
- Cho, O.H., Shin, H.M., Miele, L., Golde, T.E., Fauq, A., Minter, L.M., and Osborne, B.A. (2009). Notch regulates cytolytic effector function in CD8⁺ T cells. *J Immunol* **182**, 3380-3389.
- Choo, D.K., Murali-Krishna, K., Anita, R., and Ahmed, R. (2010). Homeostatic turnover of virus-specific memory CD8 T cells occurs stochastically and is independent of CD4 T cell help. *J Immunol* **185**, 3436-3444.
- Ciocca, M.L., Barnett, B.E., Burkhardt, J.K., Chang, J.T., and Reiner, S.L. (2012). Cutting edge: asymmetric memory T cell division in response to rechallenge. *J Immunol* **188**, 4145-4148.
- Cocchi, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C., and Lusso, P. (1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**, 1811-1815.
- Cruz-Guilloty, F., Pipkin, M.E., Djuretic, I.M., Levanon, D., Lotem, J., Lichtenheld, M.G., Groner, Y., and Rao, A. (2009). Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs. *J Exp Med* **206**, 51-59.
- Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E.W., Miller, J.D., Leslie, A.J., DePierres, C., *et al.* (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **443**, 350-354.
- Decman, V., Laidlaw, B.J., Dimenna, L.J., Abdulla, S., Mozdzanowska, K., Erikson, J., Ertl, H.C., and Wherry, E.J. (2010). Cell-intrinsic defects in the proliferative response of antiviral memory CD8 T cells in aged mice upon secondary infection. *J Immunol* **184**, 5151-5159.
- Decman, V., Laidlaw, B.J., Doering, T.A., Leng, J., Ertl, H.C., Goldstein, D.R., and Wherry, E.J. (2012). Defective CD8 T cell responses in aged mice are due to

quantitative and qualitative changes in virus-specific precursors. *J Immunol* 188, 1933-1941.

Diskin, R., Scheid, J.F., Marcovecchio, P.M., West, A.P., Jr., Klein, F., Gao, H., Gnanapragasam, P.N., Abadir, A., Seaman, M.S., Nussenzweig, M.C., and Bjorkman, P.J. (2011). Increasing the potency and breadth of an HIV antibody by using structure-based rational design. *Science* 334, 1289-1293.

Dorman, S.E., Uzel, G., Roesler, J., Bradley, J.S., Bastian, J., Billman, G., King, S., Filie, A., Schermerhorn, J., and Holland, S.M. (1999). Viral infections in interferon-gamma receptor deficiency. *J Pediatr* 135, 640-643.

Du Pasquier, R.A., Kuroda, M.J., Zheng, Y., Jean-Jacques, J., Letvin, N.L., and Koralnik, I.J. (2004). A prospective study demonstrates an association between JC virus-specific cytotoxic T lymphocytes and the early control of progressive multifocal leukoencephalopathy. *Brain* 127, 1970-1978.

Dutton, R.W., Bradley, L.M., and Swain, S.L. (1998). T cell memory. *Annu Rev Immunol* 16, 201-223.

Ejnaes, M., Filippi, C.M., Martinic, M.M., Ling, E.M., Togher, L.M., Crotty, S., and von Herrath, M.G. (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 203, 2461-2472.

Elsaesser, H., Sauer, K., and Brooks, D.G. (2009). IL-21 is required to control chronic viral infection. *Science* 324, 1569-1572.

Endo, Y., Iwamura, C., Kuwahara, M., Suzuki, A., Sugaya, K., Tumes, D.J., Tokoyoda, K., Hosokawa, H., Yamashita, M., and Nakayama, T. (2011). Eomesodermin Controls Interleukin-5 Production in Memory T Helper 2 Cells through Inhibition of Activity of the Transcription Factor GATA3. *Immunity* 35, 733-745.

Finotto, S., Neurath, M.F., Glickman, J.N., Qin, S., Lehr, H.A., Green, F.H., Ackerman, K., Haley, K., Galle, P.R., Szabo, S.J., *et al.* (2002). Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 295, 336-338.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales, F.L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., *et al.* (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347, 975-982.

Frohlich, A., Kisielow, J., Schmitz, I., Freigang, S., Shamshiev, A.T., Weber, J., Marsland, B.J., Oxenius, A., and Kopf, M. (2009). IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324, 1576-1580.

Gao, Y., Tao, J., Li, M.O., Zhang, D., Chi, H., Henegariu, O., Kaech, S.M., Davis, R.J., Flavell, R.A., and Yin, Z. (2005). JNK1 is essential for CD8+ T cell-mediated tumor immune surveillance. *J Immunol* 175, 5783-5789.

- Giorgi, J.V., Hultin, L.E., McKeating, J.A., Johnson, T.D., Owens, B., Jacobson, L.P., Shih, R., Lewis, J., Wiley, D.J., Phair, J.P., *et al.* (1999). Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* 179, 859-870.
- Goldrath, A.W., Sivakumar, P.V., Glaccum, M., Kennedy, M.K., Bevan, M.J., Benoist, C., Mathis, D., and Butz, E.A. (2002). Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. *J Exp Med* 195, 1515-1522.
- Gordon, S.M., Carty, S.A., Kim, J.S., Zou, T., Smith-Garvin, J., Alonzo, E.S., Haimm, E., Sant'Angelo, D.B., Koretzky, G.A., Reiner, S.L., and Jordan, M.S. (2011). Requirements for eomesodermin and promyelocytic leukemia zinc finger in the development of innate-like CD8+ T cells. *J Immunol* 186, 4573-4578.
- Gordon, S.M., Chaix, J., Rupp, L.J., Wu, J., Madera, S., Sun, J.C., Lindsten, T., and Reiner, S.L. (2012). The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* 36, 55-67.
- Gougeon, M.L., Lecoœur, H., Dulioust, A., Enouf, M.G., Crouvoiser, M., Goujard, C., Debord, T., and Montagnier, L. (1996). Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J Immunol* 156, 3509-3520.
- Grakoui, A., Shoukry, N.H., Woollard, D.J., Han, J.H., Hanson, H.L., Ghayeb, J., Murthy, K.K., Rice, C.M., and Walker, C.M. (2003). HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302, 659-662.
- Guidotti, L.G., and Chisari, F.V. (1996). To kill or to cure: options in host defense against viral infection. *Curr Opin Immunol* 8, 478-483.
- Hansen, S.G., Ford, J.C., Lewis, M.S., Ventura, A.B., Hughes, C.M., Coyne-Johnson, L., Whizin, N., Oswald, K., Shoemaker, R., Swanson, T., *et al.* (2011). Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. *Nature* 473, 523-527.
- Hazenbergh, M.D., Stuart, J.W., Otto, S.A., Borleffs, J.C., Boucher, C.A., de Boer, R.J., Miedema, F., and Hamann, D. (2000). T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* 95, 249-255.
- He, Y.L., Zhao, Y.R., Zhang, S.L., and Lin, S.M. (2006). Host susceptibility to persistent hepatitis B virus infection. *World J Gastroenterol* 12, 4788-4793.
- Hegel, J.K., Knieke, K., Kolar, P., Reiner, S.L., and Brunner-Weinzierl, M.C. (2009). CD152 (CTLA-4) regulates effector functions of CD8+ T lymphocytes by repressing Eomesodermin. *Eur J Immunol* 39, 883-893.

Hellerstein, M., Hanley, M.B., Cesar, D., Siler, S., Papageorgopoulos, C., Wieder, E., Schmidt, D., Hoh, R., Neese, R., Macallan, D., *et al.* (1999). Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. *Nat Med* 5, 83-89.

Hellerstein, M.K., Hoh, R.A., Hanley, M.B., Cesar, D., Lee, D., Neese, R.A., and McCune, J.M. (2003). Subpopulations of long-lived and short-lived T cells in advanced HIV-1 infection. *J Clin Invest* 112, 956-966.

Hersperger, A.R., Martin, J.N., Shin, L.Y., Sheth, P.M., Kovacs, C.M., Cosma, G.L., Makedonas, G., Pereyra, F., Walker, B.D., Kaul, R., *et al.* (2011). Increased HIV-specific CD8+ T-cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood* 117, 3799-3808.

Hertoghs, K.M., Moerland, P.D., van Stijn, A., Remmerswaal, E.B., Yong, S.L., van de Berg, P.J., van Ham, S.M., Baas, F., ten Berge, I.J., and van Lier, R.A. (2010). Molecular profiling of cytomegalovirus-induced human CD8+ T cell differentiation. *J Clin Invest* 120, 4077-4090.

Hinrichs, C.S., Spolski, R., Paulos, C.M., Gattinoni, L., Kerstann, K.W., Palmer, D.C., Klebanoff, C.A., Rosenberg, S.A., Leonard, W.J., and Restifo, N.P. (2008). IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111, 5326-5333.

Hodge, R.D., Nelson, B.R., Kahoud, R.J., Yang, R., Mussar, K.E., Reiner, S.L., and Hevner, R.F. (2012). Tbr2 is essential for hippocampal lineage progression from neural stem cells to intermediate progenitors and neurons. *J Neurosci* 32, 6275-6287.

Intlekofer, A.M., Banerjee, A., Takemoto, N., Gordon, S.M., Dejong, C.S., Shin, H., Hunter, C.A., Wherry, E.J., Lindsten, T., and Reiner, S.L. (2008). Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science* 321, 408-411.

Intlekofer, A.M., Takemoto, N., Kao, C., Banerjee, A., Schambach, F., Northrop, J.K., Shen, H., Wherry, E.J., and Reiner, S.L. (2007). Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med* 204, 2015-2021.

Intlekofer, A.M., Takemoto, N., Wherry, E.J., Longworth, S.A., Northrup, J.T., Palanivel, V.R., Mullen, A.C., Gasink, C.R., Kaech, S.M., Miller, J.D., *et al.* (2005). Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 6, 1236-1244.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. *Science* 298, 601-604.

Jamieson, B.D., Butler, L.D., and Ahmed, R. (1987). Effective clearance of a persistent viral infection requires cooperation between virus-specific Lyt2+ T cells and nonspecific bone marrow-derived cells. *J Virol* 61, 3930-3937.

Jin, H.T., Anderson, A.C., Tan, W.G., West, E.E., Ha, S.J., Araki, K., Freeman, G.J., Kuchroo, V.K., and Ahmed, R. (2010). Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 107, 14733-14738.

Jin, X., Bauer, D.E., Tuttleton, S.E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C.E., Safrit, J.T., Mittler, J., Weinberger, L., *et al.* (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 189, 991-998.

Jordan, M.B., Hildeman, D., Kappler, J., and Marrack, P. (2004). An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. *Blood* 104, 735-743.

Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., and Kaech, S.M. (2007). Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27, 281-295.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 4, 1191-1198.

Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Olsen, K.J., Podack, E.R., Zinkernagel, R.M., and Hengartner, H. (1994a). Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369, 31-37.

Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994b). Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 265, 528-530.

Kao, C., Oestreich, K.J., Paley, M.A., Crawford, A., Angelosanto, J.M., Ali, M.A., Intlekofer, A.M., Boss, J.M., Reiner, S.L., Weinmann, A.S., and Wherry, E.J. (2011). Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection. *Nat Immunol* 12, 663-671.

Kasprowicz, V., Schulze Zur Wiesch, J., Kuntzen, T., Nolan, B.E., Longworth, S., Berical, A., Blum, J., McMahon, C., Reyor, L.L., Elias, N., *et al.* (2008). High level of PD-1 expression on hepatitis C virus (HCV)-specific CD8+ and CD4+ T cells during acute HCV infection, irrespective of clinical outcome. *J Virol* 82, 3154-3160.

Kawamura, A., Koshida, S., and Takada, S. (2008). Activator-to-repressor conversion of T-box transcription factors by the Ripply family of Groucho/TLE-associated mediators. *Mol Cell Biol* 28, 3236-3244.

Kinjo, I., Gordon, S.M., Intlekofer, A.M., Dowdell, K., Mooney, E.C., Caricchio, R., Grupp, S.A., Teachey, D.T., Rao, V.K., Lindsten, T., and Reiner, S.L. (2010). Cutting edge: Lymphoproliferation caused by Fas deficiency is dependent on the transcription factor eomesodermin. *J Immunol* 185, 7151-7155.

- Klavinskis, L.S., Geckeler, R., and Oldstone, M.B. (1989). Cytotoxic T lymphocyte control of acute lymphocytic choriomeningitis virus infection: interferon gamma, but not tumour necrosis factor alpha, displays antiviral activity in vivo. *J Gen Virol* 70 (Pt 12), 3317-3325.
- Koch, U., and Radtke, F. (2011). Mechanisms of T cell development and transformation. *Annu Rev Cell Dev Biol* 27, 539-562.
- Kogawa, K., Lee, S.M., Villanueva, J., Marmer, D., Sumegi, J., and Filipovich, A.H. (2002). Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. *Blood* 99, 61-66.
- Kwon, G.S., and Hadjantonakis, A.K. (2007). Eomes::GFP-a tool for live imaging cells of the trophoblast, primitive streak, and telencephalon in the mouse embryo. *Genesis* 45, 208-217.
- Ladell, K., Hellerstein, M.K., Cesar, D., Busch, R., Boban, D., and McCune, J.M. (2008). Central memory CD8+ T cells appear to have a shorter lifespan and reduced abundance as a function of HIV disease progression. *J Immunol* 180, 7907-7918.
- Lau, L.L., Jamieson, B.D., Somasundaram, T., and Ahmed, R. (1994). Cytotoxic T-cell memory without antigen. *Nature* 369, 648-652.
- Lechner, F., Gruener, N.H., Urbani, S., Uggeri, J., Santantonio, T., Kammer, A.R., Cerny, A., Phillips, R., Ferrari, C., Pape, G.R., and Klenerman, P. (2000). CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur J Immunol* 30, 2479-2487.
- Leist, T.P., Eppler, M., and Zinkernagel, R.M. (1989). Enhanced virus replication and inhibition of lymphocytic choriomeningitis virus disease in anti-gamma interferon-treated mice. *J Virol* 63, 2813-2819.
- Lewis, D.E., Tang, D.S., Adu-Oppong, A., Schober, W., and Rodgers, J.R. (1994). Anergy and apoptosis in CD8+ T cells from HIV-infected persons. *J Immunol* 153, 412-420.
- Lewis, M.D., Miller, S.A., Miazgowiec, M.M., Beima, K.M., and Weinmann, A.S. (2007). T-bet's ability to regulate individual target genes requires the conserved T-box domain to recruit histone methyltransferase activity and a separate family member-specific transactivation domain. *Mol Cell Biol* 27, 8510-8521.
- Li, Q.Y., Newbury-Ecob, R.A., Terrett, J.A., Wilson, D.I., Curtis, A.R., Yi, C.H., Gebuhr, T., Bullen, P.J., Robson, S.C., Strachan, T., *et al.* (1997). Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet* 15, 21-29.
- Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370, 650-652.

Lugo-Villarino, G., Maldonado-Lopez, R., Possemato, R., Penaranda, C., and Glimcher, L.H. (2003). T-bet is required for optimal production of IFN-gamma and antigen-specific T cell activation by dendritic cells. *Proc Natl Acad Sci U S A* 100, 7749-7754.

Ly, K.N., Xing, J., Klevens, R.M., Jiles, R.B., Ward, J.W., and Holmberg, S.D. (2012). The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. *Ann Intern Med* 156, 271-278.

Lynch, H.E., Goldberg, G.L., Chidgey, A., Van den Brink, M.R., Boyd, R., and Sempowski, G.D. (2009). Thymic involution and immune reconstitution. *Trends Immunol* 30, 366-373.

Macintyre, A.N., Finlay, D., Preston, G., Sinclair, L.V., Waugh, C.M., Tamas, P., Feijoo, C., Okkenhaug, K., and Cantrell, D.A. (2011). Protein kinase B controls transcriptional programs that direct cytotoxic T cell fate but is dispensable for T cell metabolism. *Immunity* 34, 224-236.

Manjunath, N., Shankar, P., Wan, J., Weninger, W., Crowley, M.A., Hieshima, K., Springer, T.A., Fan, X., Shen, H., Lieberman, J., and von Andrian, U.H. (2001). Effector differentiation is not prerequisite for generation of memory cytotoxic T lymphocytes. *J Clin Invest* 108, 871-878.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M., and Albrecht, J.K. (2001). Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 358, 958-965.

Matloubian, M., Concepcion, R.J., and Ahmed, R. (1994). CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68, 8056-8063.

Matloubian, M., Kolhekar, S.R., Somasundaram, T., and Ahmed, R. (1993). Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 67, 7340-7349.

Matloubian, M., Somasundaram, T., Kolhekar, S.R., Selvakumar, R., and Ahmed, R. (1990). Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J Exp Med* 172, 1043-1048.

Matsuzaki, J., Gnjjatic, S., Mhawech-Fauceglia, P., Beck, A., Miller, A., Tsuji, T., Eppolito, C., Qian, F., Lele, S., Shrikant, P., *et al.* (2010). Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. *Proc Natl Acad Sci U S A* 107, 7875-7880.

McCune, J.M., Hanley, M.B., Cesar, D., Halvorsen, R., Hoh, R., Schmidt, D., Wieder, E., Deeks, S., Siler, S., Neese, R., and Hellerstein, M. (2000). Factors influencing T-cell turnover in HIV-1-seropositive patients. *J Clin Invest* 105, R1-8.

- McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S., and Albrecht, J.K. (1998). Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 339, 1485-1492.
- McHutchison, J.G., Lawitz, E.J., Shiffman, M.L., Muir, A.J., Galler, G.W., McCone, J., Nyberg, L.M., Lee, W.M., Ghalib, R.H., Schiff, E.R., *et al.* (2009). Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med* 361, 580-593.
- McMahan, R.H., Golden-Mason, L., Nishimura, M.I., McMahon, B.J., Kemper, M., Allen, T.M., Gretch, D.R., and Rosen, H.R. (2010). Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest* 120, 4546-4557.
- Meyaard, L., Otto, S.A., Jonker, R.R., Mijster, M.J., Keet, R.P., and Miedema, F. (1992). Programmed death of T cells in HIV-1 infection. *Science* 257, 217-219.
- Micallef, J.M., Kaldor, J.M., and Dore, G.J. (2006). Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J Viral Hepat* 13, 34-41.
- Migueles, S.A., Laborico, A.C., Shupert, W.L., Sabbaghian, M.S., Rabin, R., Hallahan, C.W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., *et al.* (2002). HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 3, 1061-1068.
- Mohri, H., Perelson, A.S., Tung, K., Ribeiro, R.M., Ramratnam, B., Markowitz, M., Kost, R., Hurley, A., Weinberger, L., Cesar, D., *et al.* (2001). Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J Exp Med* 194, 1277-1287.
- Moskophidis, D., Cobbold, S.P., Waldmann, H., and Lehmann-Grube, F. (1987). Mechanism of recovery from acute virus infection: treatment of lymphocytic choriomeningitis virus-infected mice with monoclonal antibodies reveals that Lyt-2+ T lymphocytes mediate clearance of virus and regulate the antiviral antibody response. *J Virol* 61, 1867-1874.
- Mueller, S.N., and Ahmed, R. (2009). High antigen levels are the cause of T cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 106, 8623-8628.
- Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8, 177-187.
- Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* 286, 1377-1381.

- Naiche, L.A., Harrelson, Z., Kelly, R.G., and Papaioannou, V.E. (2005). T-box genes in vertebrate development. *Annu Rev Genet* 39, 219-239.
- Nakamoto, N., Cho, H., Shaked, A., Olthoff, K., Valiga, M.E., Kaminski, M., Gostick, E., Price, D.A., Freeman, G.J., Wherry, E.J., and Chang, K.M. (2009). Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS Pathog* 5, e1000313.
- Nakamoto, N., Kaplan, D.E., Coleclough, J., Li, Y., Valiga, M.E., Kaminski, M., Shaked, A., Olthoff, K., Gostick, E., Price, D.A., *et al.* (2008). Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. *Gastroenterology* 134, 1927-1937, 1937 e1921-1922.
- Nansen, A., Jensen, T., Christensen, J.P., Andreasen, S.O., Ropke, C., Marker, O., and Thomsen, A.R. (1999). Compromised virus control and augmented perforin-mediated immunopathology in IFN-gamma-deficient mice infected with lymphocytic choriomeningitis virus. *J Immunol* 163, 6114-6122.
- Novershtern, N., Subramanian, A., Lawton, L.N., Mak, R.H., Haining, W.N., McConkey, M.E., Habib, N., Yosef, N., Chang, C.Y., Shay, T., *et al.* (2011). Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell* 144, 296-309.
- Oestreich, K.J., Huang, A.C., and Weinmann, A.S. (2011). The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J Exp Med* 208, 1001-1013.
- Oestreich, K.J., Mohn, S.E., and Weinmann, A.S. (2012). Molecular mechanisms that control the expression and activity of Bcl-6 in TH1 cells to regulate flexibility with a TFH-like gene profile. *Nat Immunol* 13, 405-411.
- Pachlopnik Schmid, J., Ho, C.H., Chretien, F., Lefebvre, J.M., Pivert, G., Kosco-Vilbois, M., Ferlin, W., Geissmann, F., Fischer, A., and de Saint Basile, G. (2009). Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. *EMBO Mol Med* 1, 112-124.
- Pearce, E.L., Mullen, A.C., Martins, G.A., Krawczyk, C.M., Hutchins, A.S., Zediak, V.P., Banica, M., DiCioccio, C.B., Gross, D.A., Mao, C.A., *et al.* (2003). Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 302, 1041-1043.
- Pellegrini, M., Calzascia, T., Toe, J.G., Preston, S.P., Lin, A.E., Elford, A.R., Shahinian, A., Lang, P.A., Lang, K.S., Morre, M., *et al.* (2011). IL-7 engages multiple mechanisms to overcome chronic viral infection and limit organ pathology. *Cell* 144, 601-613.
- Persechini, P.M., Young, J.D., and Almers, W. (1990). Membrane channel formation by the lymphocyte pore-forming protein: comparison between susceptible and resistant target cells. *J Cell Biol* 110, 2109-2116.

Pham, N.L., Badovinac, V.P., and Harty, J.T. (2009). A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation. *J Immunol* 183, 2337-2348.

Phillips, R.L., Ernst, R.E., Brunk, B., Ivanova, N., Mahan, M.A., Deanehan, J.K., Moore, K.A., Overton, G.C., and Lemischka, I.R. (2000). The genetic program of hematopoietic stem cells. *Science* 288, 1635-1640.

Piacenti, F.J. (2006). An update and review of antiretroviral therapy. *Pharmacotherapy* 26, 1111-1133.

Pipkin, M.E., Sacks, J.A., Cruz-Guilloty, F., Lichtenheld, M.G., Bevan, M.J., and Rao, A. (2010). Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* 32, 79-90.

Qui, H.Z., Hagymasi, A.T., Bandyopadhyay, S., St Rose, M.C., Ramanarasimhaiah, R., Menoret, A., Mittler, R.S., Gordon, S.M., Reiner, S.L., Vella, A.T., and Adler, A.J. (2011). CD134 Plus CD137 Dual Costimulation Induces Eomesodermin in CD4 T Cells To Program Cytotoxic Th1 Differentiation. *J Immunol*.

Quigley, M., Pereyra, F., Nilsson, B., Porichis, F., Fonseca, C., Eichbaum, Q., Julg, B., Jesneck, J.L., Brosnahan, K., Imam, S., *et al.* (2010). Transcriptional analysis of HIV-specific CD8+ T cells shows that PD-1 inhibits T cell function by upregulating BATF. *Nat Med* 16, 1147-1151.

Rao, R.R., Li, Q., Odunsi, K., and Shrikant, P.A. (2010). The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity* 32, 67-78.

Raziorrouh, B., Schraut, W., Gerlach, T., Nowack, D., Gruner, N.H., Ulsenheimer, A., Zachoval, R., Wachtler, M., Spannagl, M., Haas, J., *et al.* (2010). The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology* 52, 1934-1947.

Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premisri, N., Namwat, C., de Souza, M., Adams, E., *et al.* (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 361, 2209-2220.

Ribeiro-Dos-Santos, P., Turnbull, E.L., Monteiro, M., Legrand, A., Conrod, K., Baalwa, J., Pellegrino, P., Shaw, G.M., Williams, I., Borrow, P., and Rocha, B. (2012). Chronic HIV infection affects the expression of the two transcription factors required for CD8 T cells differentiation into cytolytic effectors. *Blood*.

Rock, K.L., and Goldberg, A.L. (1999). Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu Rev Immunol* 17, 739-779.

Rode, M., Balkow, S., Sobek, V., Brehm, R., Martin, P., Kersten, A., Dumrese, T., Stehle, T., Mullbacher, A., Wallich, R., and Simon, M.M. (2004). Perforin and Fas act

together in the induction of apoptosis, and both are critical in the clearance of lymphocytic choriomeningitis virus infection. *J Virol* 78, 12395-12405.

Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., and Izpisua Belmonte, J.C. (1999). The T-box genes *Tbx4* and *Tbx5* regulate limb outgrowth and identity. *Nature* 398, 814-818.

Russ, A.P., Wattler, S., Colledge, W.H., Aparicio, S.A., Carlton, M.B., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehls, M.C., *et al.* (2000). Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* 404, 95-99.

Rutishauser, R.L., Martins, G.A., Kalachikov, S., Chandele, A., Parish, I.A., Meffre, E., Jacob, J., Calame, K., and Kaech, S.M. (2009). Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31, 296-308.

Sachsenberg, N., Perelson, A.S., Yerly, S., Schockmel, G.A., Leduc, D., Hirschel, B., and Perrin, L. (1998). Turnover of CD4+ and CD8+ T lymphocytes in HIV-1 infection as measured by Ki-67 antigen. *J Exp Med* 187, 1295-1303.

Sakuishi, K., Apetoh, L., Sullivan, J.M., Blazar, B.R., Kuchroo, V.K., and Anderson, A.C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207, 2187-2194.

Salvato, M., Borrow, P., Shimomaye, E., and Oldstone, M.B. (1991). Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. *J Virol* 65, 1863-1869.

Scheid, J.F., Mouquet, H., Feldhahn, N., Seaman, M.S., Velinzon, K., Pietzsch, J., Ott, R.G., Anthony, R.M., Zebroski, H., Hurley, A., *et al.* (2009). Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature* 458, 636-640.

Scheid, J.F., Mouquet, H., Ueberheide, B., Diskin, R., Klein, F., Oliveira, T.Y., Pietzsch, J., Fenyo, D., Abadir, A., Velinzon, K., *et al.* (2011). Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* 333, 1633-1637.

Schmitz, J.E., Kuroda, M.J., Santra, S., Sasseville, V.G., Simon, M.A., Lifton, M.A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B.J., *et al.* (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283, 857-860.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75, 163-189.

- Shin, H., Blackburn, S.D., Blattman, J.N., and Wherry, E.J. (2007). Viral antigen and extensive division maintain virus-specific CD8 T cells during chronic infection. *J Exp Med* 204, 941-949.
- Shin, H., Blackburn, S.D., Intlekofer, A.M., Kao, C., Angelosanto, J.M., Reiner, S.L., and Wherry, E.J. (2009). A role for the transcriptional repressor Blimp-1 in CD8(+) T cell exhaustion during chronic viral infection. *Immunity* 31, 309-320.
- Shoukry, N.H., Grakoui, A., Houghton, M., Chien, D.Y., Ghayeb, J., Reimann, K.A., and Walker, C.M. (2003). Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* 197, 1645-1655.
- Siegel, A.M., Heimall, J., Freeman, A.F., Hsu, A.P., Brittain, E., Brenchley, J.M., Douek, D.C., Fahle, G.H., Cohen, J.I., Holland, S.M., and Milner, J.D. (2011). A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. *Immunity* 35, 806-818.
- Stepp, S.E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P.A., Henter, J.I., Bennett, M., Fischer, A., de Saint Basile, G., and Kumar, V. (1999). Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 286, 1957-1959.
- Sullivan, B.M., Emonet, S.F., Welch, M.J., Lee, A.M., Campbell, K.P., de la Torre, J.C., and Oldstone, M.B. (2011). Point mutation in the glycoprotein of lymphocytic choriomeningitis virus is necessary for receptor binding, dendritic cell infection, and long-term persistence. *Proc Natl Acad Sci U S A* 108, 2969-2974.
- Sullivan, B.M., Juedes, A., Szabo, S.J., von Herrath, M., and Glimcher, L.H. (2003). Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci U S A* 100, 15818-15823.
- Suto, A., Wurster, A.L., Reiner, S.L., and Grusby, M.J. (2006). IL-21 inhibits IFN-gamma production in developing Th1 cells through the repression of Eomesodermin expression. *J Immunol* 177, 3721-3727.
- Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., and Glimcher, L.H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655-669.
- Szabo, S.J., Sullivan, B.M., Stemmann, C., Satoskar, A.R., Sleckman, B.P., and Glimcher, L.H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 295, 338-342.
- Takeda, K., Cretney, E., Hayakawa, Y., Ota, T., Akiba, H., Ogasawara, K., Yagita, H., Kinoshita, K., Okumura, K., and Smyth, M.J. (2005). TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver. *Blood* 105, 2082-2089.

- Takemoto, N., Intlekofer, A.M., Northrup, J.T., Wherry, E.J., and Reiner, S.L. (2006). Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8⁺ T cell differentiation. *J Immunol* 177, 7515-7519.
- Takeuchi, J.K., Koshiba-Takeuchi, K., Matsumoto, K., Vogel-Hopker, A., Naitoh-Matsuo, M., Ogura, K., Takahashi, N., Yasuda, K., and Ogura, T. (1999). Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature* 398, 810-814.
- Tan, J.T., Ernst, B., Kieper, W.C., LeRoy, E., Sprent, J., and Surh, C.D. (2002). Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8⁺ cells but are not required for memory phenotype CD4⁺ cells. *J Exp Med* 195, 1523-1532.
- Tao, J., Gao, Y., Li, M.O., He, W., Chen, L., Harvev, B., Davis, R.J., Flavell, R.A., and Yin, Z. (2007). JNK2 negatively regulates CD8⁺ T cell effector function and anti-tumor immune response. *Eur J Immunol* 37, 818-829.
- Tayade, C., Fang, Y., Black, G.P., V, A.P., Jr., Erlebacher, A., and Croy, B.A. (2005). Differential transcription of Eomes and T-bet during maturation of mouse uterine natural killer cells. *J Leukoc Biol* 78, 1347-1355.
- Thaunat, O., Granja, A.G., Barral, P., Filby, A., Montaner, B., Collinson, L., Martinez-Martin, N., Harwood, N.E., Bruckbauer, A., and Batista, F.D. (2012). Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity. *Science* 335, 475-479.
- Tinoco, R., Alcalde, V., Yang, Y., Sauer, K., and Zuniga, E.I. (2009). Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8⁺ T cell deletion and viral persistence in vivo. *Immunity* 31, 145-157.
- Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., Atkins, M.B., *et al.* (2012). Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *N Engl J Med*.
- Townsend, M.J., Weinmann, A.S., Matsuda, J.L., Salomon, R., Farnham, P.J., Biron, C.A., Gapin, L., and Glimcher, L.H. (2004). T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20, 477-494.
- Trapani, J.A., and Smyth, M.J. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2, 735-747.
- Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T.H., Chennareddi, L., Silvestri, G., Freeman, G.J., *et al.* (2009). Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 458, 206-210.
- Vezys, V., Penaloza-MacMaster, P., Barber, D.L., Ha, S.J., Konieczny, B., Freeman, G.J., Mittler, R.S., and Ahmed, R. (2011). 4-1BB signaling synergizes with programmed death ligand 1 blockade to augment CD8 T cell responses during chronic viral infection. *J Immunol* 187, 1634-1642.

Virgin, H.W., Wherry, E.J., and Ahmed, R. (2009). Redefining chronic viral infection. *Cell* 138, 30-50.

Wang, N.S., McHeyzer-Williams, L.J., Okitsu, S.L., Burris, T.P., Reiner, S.L., and McHeyzer-Williams, M.G. (2012). Divergent transcriptional programming of class-specific B cell memory by T-bet and RORalpha. *Nat Immunol* 13, 604-611.

Wei, C.J., Boyington, J.C., McTamney, P.M., Kong, W.P., Pearce, M.B., Xu, L., Andersen, H., Rao, S., Tumpey, T.M., Yang, Z.Y., and Nabel, G.J. (2010). Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science* 329, 1060-1064.

Welsch, C., Jesudian, A., Zeuzem, S., and Jacobson, I. (2012). New direct-acting antiviral agents for the treatment of hepatitis C virus infection and perspectives. *Gut* 61 Suppl 1, i36-46.

West, E.E., Youngblood, B., Tan, W.G., Jin, H.T., Araki, K., Alexe, G., Konieczny, B.T., Calpe, S., Freeman, G.J., Terhorst, C., *et al.* (2011). Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity* 35, 285-298.

Wherry, E.J. (2011). T cell exhaustion. *Nat Immunol* 12, 492-499.

Wherry, E.J., Barber, D.L., Kaech, S.M., Blattman, J.N., and Ahmed, R. (2004). Antigen-independent memory CD8 T cells do not develop during chronic viral infection. *Proc Natl Acad Sci U S A* 101, 16004-16009.

Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003a). Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77, 4911-4927.

Wherry, E.J., Ha, S.J., Kaech, S.M., Haining, W.N., Sarkar, S., Kalia, V., Subramaniam, S., Blattman, J.N., Barber, D.L., and Ahmed, R. (2007). Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27, 670-684.

Wherry, E.J., Teichgraber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., and Ahmed, R. (2003b). Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4, 225-234.

Wiehagen, K.R., Corbo, E., Schmidt, M., Shin, H., Wherry, E.J., and Maltzman, J.S. (2010). Loss of tonic T-cell receptor signals alters the generation but not the persistence of CD8+ memory T cells. *Blood* 116, 5560-5570.

Wille, A., Gessner, A., Lothar, H., and Lehmann-Grube, F. (1989). Mechanism of recovery from acute virus infection. VIII. Treatment of lymphocytic choriomeningitis virus-infected mice with anti-interferon-gamma monoclonal antibody blocks generation of virus-specific cytotoxic T lymphocytes and virus elimination. *Eur J Immunol* 19, 1283-1288.

Woo, S.R., Turnis, M.E., Goldberg, M.V., Bankoti, J., Selby, M., Nirschl, C.J., Bettini, M.L., Gravano, D.M., Vogel, P., Liu, C.L., *et al.* (2011). Immune inhibitory molecules

LAG-3 and PD-1 synergistically regulate T cell function to promote tumoral immune escape. *Cancer Res.*

Yagi, R., Junttila, I.S., Wei, G., Urban, J.F., Jr., Zhao, K., Paul, W.E., and Zhu, J. (2010). The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma. *Immunity* 32, 507-517.

Yang, Y., Xu, J., Niu, Y., Bromberg, J.S., and Ding, Y. (2008). T-bet and eomesodermin play critical roles in directing T cell differentiation to Th1 versus Th17. *J Immunol* 181, 8700-8710.

Yi, J.S., Du, M., and Zajac, A.J. (2009). A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324, 1572-1576.

Yokoyama, W.M., Kim, S., and French, A.R. (2004). The dynamic life of natural killer cells. *Annu Rev Immunol* 22, 405-429.

Zajac, A.J., Blattman, J.N., Murali-Krishna, K., Sourdive, D.J., Suresh, M., Altman, J.D., and Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188, 2205-2213.

Zhou, Q., Munger, M.E., Veenstra, R.G., Weigel, B.J., Hirashima, M., Munn, D.H., Murphy, W.J., Azuma, M., Anderson, A.C., Kuchroo, V.K., and Blazar, B.R. (2011). Coexpression of Tim-3 and PD-1 identifies a CD8⁺ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* 117, 4501-4510.

Zhou, T., Georgiev, I., Wu, X., Yang, Z.Y., Dai, K., Finzi, A., Kwon, Y.D., Scheid, J.F., Shi, W., Xu, L., *et al.* (2010). Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science* 329, 811-817.

Zhu, Y., Ju, S., Chen, E., Dai, S., Li, C., Morel, P., Liu, L., Zhang, X., and Lu, B. (2010). T-bet and eomesodermin are required for T cell-mediated antitumor immune responses. *J Immunol* 185, 3174-3183.